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PERITONEAL FIBRINOLYSIS AND INTRA-ABDOMINAL ADHESIONS

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ABSTRACT

Intra-abdominal adhesions are the commonest cause of small bowel obstruction in developed countries. Following peritoneal injury a fibrin-rich inflammatory exudate is released into the peritoneal cavity producing delicate fibrinous adhesions which are either lysed or become organised into permanent fibrous adhesions. Studies were undertaken to determine the relationship between peritoneal fibrinolysis and intra-abdominal adhesion formation.

A fibrin plate method for quantifying functional fibrinolytic activity (measured as plasminogen activating activity: PAA) of the peritoneum was established and validated. Employing this assay fibrinolytic activity was identified in both animal and human peritoneum. Inflammation was shown to significantly reduce fibrinolytic activity in human parietal and visceral peritoneum.

The timing of reduction of peritoneal fibrinolytic activity was investigated after ischaemic, bacterial and chemical peritonitis in rats. All three injuries resulted in adhesions and a similar profile of peritoneal fibrinolytic activity: an initial significant reduction of PAA lasting 24 hours followed by a rebound peak and return to basal levels at two weeks.

Using antibody inhibition techniques tissue plasminogen activator (tPA), rather than urokinase, was shown to be the

principal plasminogen activator in human peritoneum. In inflamed human peritoneum levels of tPA were unaltered but the fast acting plasminogen activator inhibitor-one (PAI-1), not present in normal peritoneum, was elaborated and associated with loss of functional fibrinolytic activity.

The peritoneal exudate after elective surgery was found to have no fibrinolytic activity but high levels of PAI-1. These results contrasted with the findings in normal peritoneum and bile.

Recombinant tPA combined with an inert slow-release gel was tested in an animal model of ischaemic adhesion formation. Gel alone reduced adhesion formation and the addition of rtPA significantly increased this effect.

These studies indicate that peritoneal insults are accompanied by reduction in peritoneal fibrinolysis and provide a unifying pathophysiological mechanism for adhesion formation. Local delivery of the fibrin-specific agent tPA is able to reduce the formation of adhesions and this may prove to be a valuable adjunct in clinical practice.

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Publications and presentations

The work described in this thesis has resulted in the following publications and presentations:

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Peritoneal fibrinolytic activity and intra-abdominal adhesions. *Lancet* 1990; **i**: 1120-1122.

Vipond MN, Whawell SA, Harbourne T, Thompson JN, Dudley HAF. Peritoneal fibrinolytic activity and adhesions. *Surgical Research Society*, Newcastle, July 1989. *British Journal of Surgery* 1989; **76**: 1335.

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Fibrinolysis in experimental peritoneal injury. *Fibrinolysis Workshop*, Leiden, February 1990.

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Intraperitoneal infection and adhesions. *Surgical Infection Society*, Antwerp, June 1990. *Surgical Research Communications* 1990; **8 (Suppl. 1)**: 43.

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Reduced peritoneal fibrinolytic activity and adhesions. *10th International Congress on Fibrinolysis*, Indianapolis, August 1990. *Fibrinolysis* 1990; **4 (Suppl. 3)**: 153.

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. The aetiology and prevention of ischaemic adhesions. *British Society of Gastroenterology*, Southampton, September 1990. *Gut* 1990; **31**: A1198-1199.

Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Adhesion prophylaxis with recombinant tissue plasminogen activator. *Surgical Research Society*, London, January 1991. *British Journal of Surgery* 1991; **78**: 744.

Abbreviations

Ab	antibody
ANOVA	analysis of variance
cps	centipoise
CFU	colony forming units
ELISA	enzyme-linked immunosorbent assay
kD	kilodalton
PA	plasminogen activator
PAA	plasminogen activating activity
PAI-1	plasminogen activator inhibitor-one
PAI-2	plasminogen activator inhibitor-two
PAI-3	plasminogen activator inhibitor-three
rtPA	recombinant tissue plasminogen activator
sc	single chain
tc	two chain
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator

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CHAPTER 1

INTRODUCTION

1.1 ADHESIONS: THE PROBLEM

1.1.1 Incidence

Intra-abdominal adhesions appear to be almost inevitable after abdominal surgery and are the commonest cause of small bowel obstruction in countries with developed medical services. Systematic studies of the overall incidence of adhesions after surgery are rare but Weibel and Majno (1973) reviewed 298 subjects with previous abdominal surgery at post-mortem and found intra-abdominal adhesions in two thirds. For subjects who had undergone multiple operations the incidence rose to 93%. A similar figure was found in a smaller study of 50 consecutive patients undergoing a second laparotomy where the incidence of adhesions was 88% (Ellis 1982).

More recently, a detailed survey of the relationship between intestinal obstruction, adhesions and previous abdominal surgery has been published (Menzies & Ellis, 1990a). In a prospective analysis of 210 patients, who had previously undergone one or more abdominal operations, 93% were found to have intra-abdominal adhesions at a subsequent laparotomy. This compared with an incidence of 10.4% in 115 patients who had never previously undergone abdominal surgery. In a retrospective survey of the same unit over a 25 year period, 261 of 28 297 (0.9%) adult general surgical admissions were for adhesive intestinal obstruction. Of 4502 laparotomies performed during this

period, 148 were for intestinal obstruction from adhesions (3.3%). Additionally, one per cent of all patients undergoing laparotomy for any cause developed intestinal obstruction due to post-operative adhesions within one year of surgery.

For the five year period from 1985 to 1990, in the Academic Surgical Unit at St. Mary's Hospital, 97 of 5110 (1.9%) admissions were for intestinal obstruction secondary to adhesions and 76% of these patients underwent urgent surgery (personal audit).

This high incidence of adhesive obstruction related to previous abdominal surgery has gradually developed as abdominal surgery has been more frequently performed. This can in part be related to improvements in surgical and anaesthetic techniques allowing what were previously inoperable abdominal conditions to be now tackled with relative safety. These improvements in surgical services have also led to abdominal herniae, in the past the commonest cause of bowel obstruction, to be now repaired electively before obstruction ensues.

1.1.2 Aetiology of intestinal obstruction

The changing aetiological pattern of intestinal obstruction can be traced over the course of the twentieth century (Table 1.1). Gibson in 1900 reviewed 1000 cases of intestinal obstruction collated from a series of papers

Table 1.1 *Pattern of intestinal obstruction this century (includes both small and large bowel)*

Author	Year	Country	n	adhesions (%)	hernia (%)	malignancy (%)
Gibson	1900	USA	1000	18	35	-
Souttar	1925	UK	3064	11	46	-
Vick	1932	UK	6892	7	48	14
McIver	1932	USA	335	30	44	10
Moss	1934	USA	511	27	41	3
Nemir	1952	USA	430	33	21	20
Perry	1955	USA	1252	31	10	27
Ellis	1982	UK	253	26	21	30
Bevan	1975	UK	414	16	19	29
Bevan	1984	UK	277	38	13	17
Nelson	1984	UK	279	31	23	30
McEntee	1987	UK	228	32	25	26

published between 1888 and 1898. In this collective series 35% were due to strangulated hernias and 18% secondary to intra-abdominal adhesions. Souttar (1925) and subsequently Vick (1932) in two consecutive series from teaching hospitals in England and Scotland collected 10 000 cases of intestinal obstruction in which adhesions accounted for only 11% and 7% of cases respectively. This compared with 46% and 49% respectively for abdominal herniae.

In the United States of America (USA) two series published in the same decade (McIver 1932, Moss & McFetridge 1934) confirmed that hernia was the commonest cause of obstruction (41-44% of cases) but that the incidence of adhesions was between 27% and 30%, a higher figure than that reported in the United Kingdom (UK). In the latter half of this century adhesions have superseded hernia as the commonest cause of intestinal obstruction. Perry, Smith & Yonehiro (1955) reported adhesions as the cause in 31% compared with hernia in 10%. In the UK this changing pattern has been observed within one hospital over a 20 year period (Bevan 1984). From 1960-1975 adhesions accounted for only 16% of intestinal obstruction. From 1976-1980 this figure had risen to 38% with hernia accounting for only 13%. These figures are confirmed most recently by Nelson & Ellis (1984) and McEntee et al (1987) with adhesions the cause in one third and hernia in one quarter of patients with intestinal obstruction.

In those series where the figures for small bowel obstruction alone are available (Table 1.2) the incidence of adhesions as the cause is two thirds (Räf 1969a, Playforth, Holloway & Griffen 1970, Stewardson, Bombeck & Nyhus 1978). This compares with figures from earlier this century where the predominant cause of small bowel obstruction alone is hernia (61%) with adhesions accounting for only 12% (Vick 1932). It is evident from these studies that, in countries with a developed medical service, adhesions are now the commonest cause of intestinal obstruction overall and, for the relatively mobile small bowel, are the cause of obstruction in nearly three quarters of patients.

This rising incidence of adhesions as a cause of obstruction mirrors the development of surgical advances in Western Europe and the USA. It is interesting to compare the figures with those currently reported from third world countries whose surgical services are still evolving and lag behind the developed world. In a series of 794 patients from Kampala Uganda (McAdam 1961), the incidence of strangulated hernia was 75% compared with only 4% for adhesions. Similarly in Ibadan Nigeria (Cole 1965), the relative proportions were hernia 35% and adhesions 10%. Gill and Eggleston (1965) found that of 147 patients in the Punjab India with acute intestinal obstruction, hernia was the cause in 27% and adhesions in 15%. In two more recent

Table 1.2 *Adhesions and small bowel obstruction*

Author	Year	Country	n	adhesions (%)	hernia (%)	malignancy (%)
Western Europe and USA						
Vick	1932	UK	4080	12	61	1
Rãf	1969a	Sweden	2295	64	-	-
Playforth	1970	USA	111	54	23	0
Laws	1976	USA	465	69	8	9
Stewardson	1978	USA	238	64	24	7
Bizer	1981	USA	405	74	8	9
Brolin	1987	USA	311	73	7	14
Developing Countries						
McAdam	1961	Uganda	794	4	75	1
Brooks	1966	Jamaica	250	23	25	5
Badoe	1968	Ghana	782	10	78	1
Chiedozi	1980	Nigeria	316	11	65	0.3
Tanphiphet	1987	Thailand	605	53	-	-

studies, hernia was found to be the underlying cause in three quarters of all small bowel obstructions (Badoe 1968, Chiedozi, Aboe & Piserchia 1980). However, as surgical services develop in such countries, the incidence of adhesive small bowel obstruction increases markedly. This is demonstrated in a University Hospital in Thailand (Tanphiphet, Chittmittrapap & Prasapsunti 1987) where adhesions accounted for 53% of small bowel obstruction. Similarly, two series from Malaysia (Ti & Yong 1976, Lee & Ong 1991) have shown this effect in one country: in 1976 hernia was the commonest cause of bowel obstruction; in 1991 this had been superseded by adhesions.

1.1.3 Aetiology of adhesions

The underlying cause of intra-abdominal adhesions leading to intestinal obstruction has been looked at in several series with the most frequent association being previous abdominal surgery (Table 1.3). Becker (1952) observed that in 412 patients operated on for adhesive obstruction previous surgery accounted for 90% of adhesions. Nemir (1952) analysed the records of 142 patients and found that: 103 patients (73%) had undergone previous abdominal operations; 29 had previous inflammatory disease; eight had congenital adhesions; and two were unclassified. In a review of 388 cases of adhesive obstruction (Perry, Smith & Yonehiro 1955): 79% occurred

Table 1.3 *Underlying cause of adhesions in adhesive obstruction*

Author	Year	n	previous abdo surgery (%)	inflammatory (%)	congenital (%)
Becker	1952	412	90	-	-
Nemir	1952	142	73	20	6
Perry	1955	388	79	18	11
Bevan	1984	104	88	-	-
McEntee	1987	75	81	13	4

post-operatively; 18% followed inflammatory disease; and 11% were congenital. Similarly Miller & Winfield (1959) found adhesions to be post-operative in 79%, inflammatory in 18% and congenital in 3%.

1.1.4 Adhesions after surgery

It is apparent that some intra-abdominal procedures are more likely to result in post-operative obstruction from adhesions than are others (Table 1.4). Of 63 patients with intestinal obstruction who had previous surgery (Miller & Winfield 1959), the previous operation was: appendicectomy, 29%; colo-rectal, 14%; and obstetric or gynaecological, 51%. McEntee et al (1987) found that colonic and gynaecological surgery accounted for 60% of the cases of adhesive obstruction and in the series reported by Menzies & Ellis (1990a) these two forms of surgical intervention were the predisposing operations in 68%. It is thus apparent that surgical procedures involving the peritoneal cavity in the infracolic compartment are more frequently complicated by adhesive obstruction. This is illustrated by the procedure of restorative proctocolectomy with ileal reservoir where the incidence of post-operative adhesive obstruction ranges from 13-25% depending on the length of follow up (Nicholls & Lubowski 1987, Everett 1989). This is not to say that adhesions do not follow supracolic surgery but that complications from their

Table 1.4 *Type of previous surgery and adhesion formation*

Author	Year	n	appx (%)	colon (%)	gynae (%)	chole (%)	other (%)
Miller	1959	63	29	14	51	11	0
Rāf	1969b	1477	38	6	28	7	21
Bevan	1984	104	33	9	20	11	27
McEntee	1987	75	20	20	20	5	16
Tanphiphet	1987	321	34	6	15	17	14
Menzies	1990a	80	15	39	14	5	28
Fūzūn	1991	256	38	7	17	7	27

appx = appendicectomy
colon = colorectal
gynae = gynaecological
chole = cholecystectomy

presence are less frequent. The likely explanation for this is that the mobile small bowel lies predominantly below the transverse mesocolon and, therefore, is more likely to be involved in the adhesive process after infracolic compartment surgery.

1.1.5 Complications of adhesions

In the majority of patients the formation of adhesions will cause no problems. However, if intestinal obstruction occurs, there is an associated mortality and morbidity. The mortality of intestinal obstruction has been reduced, with improved preoperative resuscitation, peri-operative management and anaesthesia, from 43% (Gibson 1900) to a range of 1-7% in modern times (Stewardson, Bombeck & Nyhus 1978, Bevan 1984, McEntee et al 1987; Table 1.5). Nevertheless, recent figures from the Office of Population Census Surveys show that small bowel obstruction, of which adhesions will cause a significant proportion, resulted in 1400 deaths in England and Wales in 1987, and approached 3000 deaths in each of the preceding two years. This mortality from adhesive obstruction, which is an otherwise benign condition, might be avoidable if the incidence of adhesions after abdominal surgery were reduced.

Surgery for adhesive obstruction will also result in morbidity, which was as high as 26% in one series

Table 1.5 *Mortality of adhesive obstruction*

Author	Year	n	urgent surgery (%)	mortality (%)
Vick	1932	505	-	33
Perry	1955	388	71	10
Miller	1959	63	68	8
Laws	1976	320	-	4
Stewardson	1978	157	71	3
Bevan	1984	104	73	7
McEntee	1987	75	29	1

(Stewardson, Bombeck & Nyhus 1978), and a hospital stay in those patients not requiring surgery. For those not undergoing surgery, the mean hospital stay was 4.6 days and for those undergoing operation 16.9 days (McEntee et al 1987). Repeated hospital admissions may be required for patients with sub-acute bowel obstruction. Thus adhesions, which cause intestinal obstruction, result in a high price shouldered both by the patient and the health service. Intra-abdominal adhesions also complicate any further abdominal surgery in patients and are a well recognised hazard to operating with an increased morbidity associated with their presence (Kirk 1987). It has been suggested that adhesions may be responsible for some instances of chronic abdominal pain following surgery though this remains to be proven (Alexander-Williams 1987). The presence of adhesions following pelvic surgery or infection is closely related to subsequent infertility in women (Caspi, Halperin & Bukovsky 1979, Holtz 1984).

Despite these problems associated with adhesion formation there are some instances when adhesions may be beneficial. Their development may wall off intraperitoneal infection resulting in an abscess and preventing diffuse peritonitis or systemic spread. Also adhesions may form to an intestinal anastomosis helping to contain a leak should this have occurred.

1.1.6 Summary

This review indicates that the incidence of adhesions has increased markedly throughout the 20th Century in direct relationship to the number of surgical procedures performed on the colon and pelvis. This has resulted in the management of intestinal obstruction secondary to adhesions becoming an important part of the surgeon's workload. In the UK adhesive obstruction now results in 1% of all general surgical admissions and 3% of all laparotomies (Menzies & Ellis 1990a). The increased incidence of adhesive obstruction and the morbidity and mortality that ensues demands further study of the mechanisms by which adhesions form and new approaches to their controlled prevention.

1.2 PERITONEAL STRUCTURE

The peritoneum is a serous membrane that lines the abdominal cavity and the intra-abdominal viscera. Etymologically, "peritoneum" is derived from the Greek, *peritonaion*, meaning "stretch around" which perfectly describes the arrangement of this thin membrane. For convenience the peritoneum is divided into the parietal peritoneum which clothes the anterior and posterior abdominal walls, and the visceral peritoneum, a

continuation of the parietal peritoneum, which leaves the posterior wall to invest the intra-abdominal viscera (Last 1978). The peritoneum is a derivative of the mesoderm and the largest serous membrane of the body with a surface area of approximately 2 m^2, roughly equivalent to that of the skin (Bouchet, Voilin & Yver 1989). It consists of a single layer of mesothelial cells covered by a 5 µm film of liquid, the peritoneal fluid.

On scanning electron microscopy the mesothelium appears as a continuous surface covered by numerous microvilli. The microvilli vary considerably: lengths range from 0.04µm to 0.08µm and density from 20 to 60 per μm^2 (Andrews & Porter 1973). The functional significance of these microvilli is not clear although it is likely that they increase surface area and facilitate exchange across the peritoneal membrane (Odor 1954). They may also act, together with serous exudate, to reduce functional resistance. In support of this theory is the higher density of microvilli found on peritoneum covering very mobile organs (Andrews and Porter 1973).

The mesothelium itself consists of flattened cells lying in close apposition on a continuous and homogeneous basement membrane. The intercellular space between adjacent cells is interrupted by tight junctions but there are no tripartite junctional complexes as seen in epithelium (Eskeland & Kjaerheim 1966a). The mesothelial

cell contains small rod-shaped mitochondria and scanty smooth endoplasmic reticulum. The most conspicuous cytoplasmic structures are pinocytotic vesicles which are able to communicate with the extracellular space (Eskeland & Kjaerheim 1966a). The basement membrane is separated from adjacent connective tissue by a membrane consisting of a inner and outer dense line separated by a lighter area, the three structures measuring about 50 nm in width (Baradi & Rao 1976). Beneath the basal lamina is a layer of very fine connective tissue fibrils.

The interstitium consists of loose tissue made up of large bundles of collagenous fibrils separated by large structureless areas. Fibroblasts may be observed in this region and the interstitium also contains lymphatic and blood vessels. The minimum distance separating blood vessels from the surface of the human peritoneum is 5-20 µm (Bundgaard & Frokjaer-Jenson 1982). There are gradual structural variations throughout the microvasculature. Capillaries and post-capillary venules are made up of a single layer of endothelial cells lying on a basement membrane in close proximity to the mesothelium (Gotloib et al 1983) and probably play an important role in the regulation of fluid movement between the peritoneal cavity and blood stream (Nolph et al 1981).

Peritoneal structure is thus well suited to its role as a lubricated protective lining which has the ability to

synthesise, absorb and secrete. The peritoneum plays a central part in the development of adhesions - further understanding of this process requires knowledge of the peritoneal response to injury and mechanism of healing.

1.3 PERITONEAL INJURY AND HEALING

When the peritoneum is stimulated or injured by a physical, chemical or biological agent an acute inflammatory reaction ensues. There is dilatation of the sub-mesothelial vasculature with increase in the permeability of the capillary walls and margination of leucocytes. Exudation of protein-rich fluid and neutrophils follows leading to oedema of the peritoneum. Fibrinogen is one of the main plasma proteins and a principal component of the inflammatory exudate. Within the inflammatory exudate fibrinogen is converted to insoluble fibrin which is deposited in the inflamed tissue. This fibrinous exudate can be seen covering the surface of the peritoneal wound for the first 48 hours following injury (Hubbard et al 1967, Raftery 1973a). Within these fibrin strands are the cellular components of the acute inflammatory reaction: macrophages, monocytes, eosinophils and polymorphonuclear leucocytes (Ellis, Harrison & Hugh

1965, Eskeland 1966a).

During the period of acute inflammation and fibrin deposition the damaged mesothelium begins to repair. The precise mechanism by which mesothelial regeneration takes place is however subject to debate. Von Dembowski (1888) and Hertzler (1919) showed that when a defect was created in the parietal peritoneum it healed quickly without ingrowth from the margins of the wound. This is unlike a defect in squamous epithelium where healing is primarily centripetal. There has been much debate as to the mechanism of peritoneal healing and the origin of the cells forming the new mesothelium.

1.3.1 Mesothelial implantation

Cameron, Hasen & De (1957) observed that when a defect was produced in the visceral peritoneum of the liver tiny islets of new cells were present on the surface of the wound. They believed, from the morphology, that these were mesothelial cells which, after becoming detached from opposing peritoneum in contact with the raw surface of the wound, acted as free peritoneal grafts. By covering the peritoneal defect with polythene they observed that healing was delayed, although the wound did ultimately produce a new mesothelium. This was interpreted as confirming that a free peritoneal graft was necessary for rapid peritoneal healing and the delayed healing observed beneath the

polythene sheet was due to a combination of centripetal healing and slow transformation of mononuclear cells in the wound. The concept of detachment of mesothelial cells and implantation in the peritoneal wound also received support from Johnson & Whitting (1962) and Bridges & Whitting (1964). However the former also conceded that mononuclear cells and macrophages on the wound surface were probably able to transform into mesothelial cells. If mesothelium from the adjacent peritoneum were able to detach and then attach to the peritoneal wound one would expect these cells to be present in free peritoneal fluid in large numbers. However Eskeland & Kjaerheim (1966a) found no mesothelial cells in rat peritoneal fluid following injury but there were large numbers of macrophages (50 000 - 60 000 cells/ml). Similarly Raftery (1973b) found very few free mesothelial cells in peritoneal fluid after injury and, using trypan blue vital staining, the cells present were almost all non-viable. This finding is further confirmed by work showing that the predominant cell in peritoneal fluid after peritonitis is the neutrophil with the mesothelial cell accounting for less than 1% of the cell population (Stewart, Holloway & Isbister 1984). It is thus unlikely that free mesothelial cells make a major contribution to the healing process.

1.3.2 Fibroblast transformation

Robbins, Brunschwig & Foote (1949) investigated the healing of parietal peritoneum in dogs and observed that the peritoneal wound healed within 4-7 days and not from the edges. This feature of mesothelial healing was confirmed by Williams (1955). On the basis that healing occurred over the whole surface of the wound it was suggested that the new mesothelium arose from the underlying connective tissue. A detailed light microscopical study by Ellis, Harrison & Hugh (1965) showed that adult rat peritoneum healed rapidly with a continuous layer of flattened cells appearing over the whole surface by 3-5 days. There was no difference in the rate of healing with the size of the wound. Nevertheless they and others (Johnson & Whitting 1962) did observe mitoses and spreading of the mesothelium at the periphery of the wound to indicate some contribution by centripetal healing.

Ellis, Harrison & Hugh (1965) showed a two-phase process of mesothelial healing and this process has been more recently observed by Elkins et al (1987). In the first 48 hours the parietal peritoneal defect was filled with an inflammatory exudate of monocytes, polymorphonuclear cells and eosinophils in a fibrinous exudate. By 72 hours these cells had mostly disappeared and the wound largely covered by a layer of fibroblasts with numerous mitoses. By 5 days this layer was

indistinguishable from adjacent mesothelium and had few mitoses. They concluded that mesothelial regeneration occurred from underlying cells, predominantly fibroblasts. This argument was supported by the general principle that cells of mesodermal origin repair fundamentally by fibroblast transformation in mammals.

1.3.3 Macrophage metaplasia

Eskeland (1966a) used Häutchen preparations of healing peritoneum to study the source of the regenerating mesothelium in peritoneal defects in rats. In such a preparation, sections of the wound are cut parallel to its surface allowing the cells of the healing process to be studied *en face*. Eskeland also observed a two-phase process with an initial wave of acute inflammatory cells within a fibrin mesh. This exudate gradually disappeared and, from 3-5 days, was replaced by rounded cells with high mitotic activity which gradually assumed the characteristics of normal mesothelium. He concluded that the rounded cells were peritoneal macrophages and the new mesothelium was derived from metaplasia of these undifferentiated macrophages in the wound. Using electron microscopy Eskeland & Kjaerheim (1966a) further defined these cellular changes. In the first 4 hours after injury they found the cellular exudate was composed mainly of macrophages within fibrin strands. Over the next 48 hours

cellular content increased with the presence of polymorphonuclear leucocytes. By 4-6 days there were elongated, flat surface cells with abundant endoplasmic reticulum but no tight junctions or basement membrane. Definite mesothelial cells were observed at 6 days. They concluded that macrophages differentiated into the new mesothelium and suggested that the source of macrophages was from circulating peritoneal fluid. This was supported by the observation that the macrophage was both the predominant cell in normal peritoneal fluid and appeared in the surface of the wound at 4 hours when, if the macrophage was from inflammatory exudate, one would expect a high population of neutrophils. In further studies autologous rat peritoneal fluid was cultured in intraperitoneal diffusion chambers (Eskeland 1966b, Eskeland & Kjaerrheim 1966b). The inside of the chamber became coated with macrophages and within a week appearances had changed to fibroblasts forming a complete surface lining of the inner wall of the diffusion chamber. It was suggested that macrophages transform to fibroblasts which then undergo metaplasia to mesothelial cells.

Thus there are conflicting theories as to whether macrophages or fibroblasts transform to produce the new mesothelium or this process is part of a continuum. Raftery (1973a) incorporated 0.79μm polystyrene spheres

into macrophages within the peritoneal cavity and then observed peritoneal healing by the use of Häutchen preparations. The spheres were observed within macrophages and polymorphs on the wound surface up to 48 hours but not in subperitoneal fibroblasts. As in the other studies the flattened mesothelium formed over the next few days. However none of the surface cells contained the microspheres although they were still present in macrophages in the depth of the wound and surrounding peritoneal fluid for up to two weeks. At no point were microspheres seen in newly formed mesothelial cells providing evidence against the theory that peritoneal macrophages are transformed into mesothelial cells via fibroblasts.

1.3.4 Circulating precursor

A further suggestion for the origin of the new mesothelium is a circulating precursor stem cell derived from the bone marrow (Wagner et al 1982). This was based on studies of a proliferating, transplantable rat mesothelioma. However this theory was invalidated by showing that whole body irradiation, sufficient to depress the bone marrow, did not prevent mesothelial healing (Whittaker & Papadimitriou 1985). It is possible that some cells circulating prior to irradiation could provide a source for new mesothelium but this is unlikely.

1.3.5 Summary

Overall it is likely that mesothelial healing depends on a number of factors. The concept of a circulating precursor can probably be dismissed. Observations that large wounds heal as quickly as small ones indicates that healing does not occur exclusively from the edges. Free mesothelial grafts can occur but serial histological studies and observations on the cell population and viability of mesothelial cells in peritoneal fluid indicate that this is a minor mechanism.

All serial studies of mesothelial injury have shown that healing occurs in two stages. In the first there is a marked inflammatory exudate of neutrophils, macrophages, monocytes and eosinophils entrapped in a fibrinous mesh. This is followed at 48 hours by clearance of the exudate and the appearance of flattened cells over the wound surface which gradually assume the morphology of a new mesothelium at 3-6 days. It appears most likely that these surface cells are derived from fibroblasts. The macrophage probably plays an important role: firstly, in the phagocytosis of cellular debris and secondly, secretion of a factor which is known to lead to fibroblast differentiation (Leibovich & Ross 1976). *In vitro* studies have shown that peak secretion of macrophage-derived fibroblast growth factor (MDFGF) occurs at 4-7 days after tissue injury. This coincides with the change in

appearance of fibroblasts to mesothelium and MDFGF has been shown *in vitro* to stimulate transformation and proliferation of fibroblasts (Orita et al 1986). Although not yet shown *in vivo* to be important MDFGF could lead to fibroblast metaplasia and the formation of a new mesothelium. Additional healing from the wound edge and implantation of viable mesothelial cells probably also plays a role in rapid healing and recovery of mesothelial function.

1.4 PATHOPHYSIOLOGY OF ADHESION FORMATION

Audit of adhesive intestinal obstruction in humans, as outlined in Section 1.1, indicates that the commonest predisposing causes are previous surgery (73-80%), bacterial or chemical inflammation (18-20%) and congenital bands (6-11%), (Nemir 1952, Perry, Smith & Yonehiro 1955, Bevan 1984, McEntee et al 1987). Thus a variety of mechanisms of peritoneal injury may lead to adhesion formation. To determine the sequence of events in the pathophysiology of adhesion formation it has proved necessary to undertake animal studies.

1.4.1 Time sequence of adhesion formation

Adhesion formation is a rapid process. Hertzler (1915) showed that as soon as ten minutes after trauma a fibrinous network begins to develop and is fully formed after two hours. This finding has recently been confirmed by Menzies & Ellis (1990b). The process of adhesion formation has been observed macroscopically using a 6 x 8 cm plexiglass window sutured in the anterior abdominal wall of dogs (Jackson 1958). Within the first three hours there was marked capillary dilatation within the injured peritoneum, fluid exudate and outpouring of fibrin. By 4-6 hours fibrinous adhesions were visible between damaged bowel surfaces or damaged parietal peritoneum and omentum. If these fibrinous adhesions persisted beyond five days permanent fibrous (mature) adhesions were invariably found. The introduction of bacterial contamination led to more rapid adhesion formation and the localisation of pus as abscesses. Blood however was slowly absorbed and not observed to play a role in adhesion formation.

1.4.2 Ischaemic injury

Detailed observation of the adhesive process to devitalised segments of rat small intestine was undertaken by Ellis (1962). In this study the small bowel vasculature was divided to devitalise segments of small intestine ranging in length from 2.5 to 9 cm. Adhesions formed in

62/65 (95%) animals and the number of adhesions was proportional to the length of devitalised bowel. All adhesions contained blood vessels but segments longer than 6 cm all became gangrenous. If a 2.5 cm length of devitalised bowel was wrapped in polythene preventing the adhesions from reaching the bowel surface this short segment became gangrenous. This led Ellis to conclude that the primary stimulus for adhesion formation was ischaemia and adhesions themselves were a process of revascularising devitalised tissue.

Additional studies by Ellis confirmed ischaemia to be a powerful stimulus to adhesion formation. With parietal peritoneal defects of 1-3 cm^2 no adhesions formed unless the defect had been closed by suturing. This was shown not to be a foreign body reaction by the absence of adhesions to sutures placed loosely through the peritoneum. Similarly ischaemia induced by a crushed area of peritoneum or ligated button of peritoneum formed adhesions.

Further support for adhesions being an attempt at revascularisation came from studies by Myllärniem & Karppinen (1968) who stripped a segment of visceral and parietal peritoneum. Fibrinous adhesions formed by three hours and were followed by the development of fibrous mature adhesions which showed blood vessels on angiography.

The observation that vascular adhesions form to areas of ischaemia is supported by clinical observation:

adhesions are often found to a bowel anastomosis or the inner aspect of a laparotomy wound where suturing produces localised areas of tissue ischaemia. Also tumours which have undergone ischaemic degeneration or ovarian cysts which have undergone torsion are usually found wrapped in dense adhesions. However ischaemia alone fails to explain the adhesions found after mechanical trauma, chemical injury or bacterial contamination.

1.4.3 Mechanical injury

Ryan, Grobéty & Majno (1971) studied the effect of a mild mechanical injury on adhesion formation. Loss of the caecal mesothelium in rats was produced by drying in air for five minutes. This injury alone did not produce adhesions but when combined with 2 ml of fresh blood resulted in adhesions in all animals. Microscopically the caecal drying led to patchy loss of the mesothelial surface and some fibrin exudate. The addition of blood led to a clot forming on the denuded caecal surface and fibrinous adhesions to adjacent peritoneum or omentum. Adhesions were not produced with blood alone or with plasma or defibrinated blood combined with caecal injury. They suggested that the essential component for adhesion formation was fibrin which, if able to remain until fibroblasts invaded the injured area/blood clot, became organised. They concluded that *"adhesions were the result*

of organisation of fibrin between opposing surfaces".

The most detailed light and electron microscopic studies of adhesion formation were provided by Milligan & Raftery (1974). These studies used an abrasive injury to the caecum and parietal peritoneum. Adhesions formed in 74/75 rats. In the first two days there was a marked exudate with neutrophils, macrophages and eosinophils in a fibrin matrix. At three days the fibrin became more fibrillar and the predominant cellular component was macrophages and fibroblasts with collagen production. Over the next three days, fibrin diminished and there was increased collagen production. At two weeks collagen was present in discrete bundles with parallel fibroblasts lying between these collagen bundles. This represented mature fibrous adhesions and appearances remained stable over the next two months.

1.4.4 Summary

The above studies indicate that the incorporation of collagen, following fibroblast invasion, in the fibrinous exudate leads to permanent adhesions (Figure 1.1). On the basis of these findings attempts have been made to reduce adhesion formation in both animal models and clinical studies.

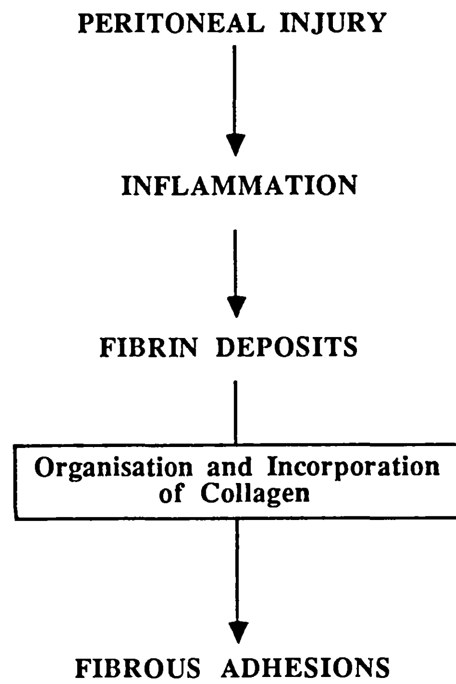

Figure 1.1 *A proposed pathway for adhesion formation*

1.5 PREVENTION OF ADHESIONS

Over the course of this century a wide variety of mechanical methods and pharmacological agents have been used in an attempt to prevent adhesion formation. In many instances agents have been employed with no scientific basis for their potential efficacy and consequently results have been disappointing. Ellis (1971) has classified the possible mechanisms by which researchers have approached the problem of adhesion prevention:

1. prevention of fibrin deposition
2. removal of fibrin exudate
3. separation of peritoneal surfaces
4. inhibition of fibroblastic proliferation
5. controlled adhesion formation

1.5.1 Prevention of fibrin deposition

Anticoagulants were first proposed to prevent adhesion formation by Lehman & Boys (1940). The rationale was that such substances would prevent the conversion of fibrinogen to fibrin within the peritoneal cavity as occurs within the vascular system. In these studies a single dose of intraperitoneal heparin (750-3000 IU) completely prevented the reformation of surgically divided adhesions in rabbits and reduced them by by 75% in dogs. However, results for

20% of the rabbits and 63% of the dogs studied were omitted for unspecified reasons. Another anticoagulant, dicumarol, was also found to be effective but with a high mortality secondary to haemorrhage (White 1949). Subsequent studies with heparin have also reported a high incidence of haemorrhage. Recently both intraperitoneal and subcutaneous heparin has been shown to reduce adhesions to sites of microsurgical anastomosis in rats (Al-Chalabi & Otubo 1987). No data for morbidity were given in this study and heparin was found to be ineffective in preventing adhesions secondary to bacterial peritonitis.

Fibrin deposition may also be reduced by ancrod, derived from the venom of the Malayan pit viper, which specifically depletes the plasma fibrinogen available for conversion to fibrin. However, in a mechanical trauma model in dogs, systemic ancrod delivered for 48 hours after trauma, failed to reduce omental adhesions to the areas of trauma despite evidence of systemic fibrinogen depletion (Buckman et al 1975). It was suggested that the duration of ancrod delivery may have been too short as systemic defibrogenation would need to be maintained until exudation of plasma from the damaged mesothelium ceased or there was a return of local fibrinolytic activity.

1.5.2 Removal of fibrin exudate

The common pathway to the formation of permanent

adhesions is the organisation of fibrin into fibrous tissue. This has led a number of workers to employ methods to remove the fibrinous exudate. A variety of approaches have been advocated including washing away the deposited fibrin, digestion with proteolytic enzymes and the use of specific fibrinolytic agents.

Peritoneal lavage to wash away fibrinous exudate has been attempted with a number of agents but the rapid absorption of such solutions make their efficacy unlikely. It has been shown, in the rat, that a volume of plasma equal to half the normal blood volume is absorbed across the peritoneal cavity every three to five hours (Courtice & Simmonds 1954). The failure of this approach has been demonstrated most recently using saline irrigation of injured rat serosa (Larsson & Perbeck 1986). Protein exudate was measured after intravenous administration of fluore-isothiocyanate-dextran and then measuring fluorescence intensity from injured serosa and comparing this with normal serosa. The total exudate of protein was actually increased by saline irrigation and adhesion formation unaffected. Other crystalloid solutions are similarly ineffective (Holtz 1984).

Dextran solutions have also been widely used in the belief that they possess some fibrinolytic properties and the high molecular weight delays absorption. diZerega & Hodgen (1980) reported that 32% Dextran 70 remained in the

peritoneal cavity for up to five days after instillation. A number of studies have used this high molecular weight Dextran with variable results; although subjective adhesion scores were often lower there was no universal reduction in the number of adhesions (Pope 1914, David & Sparks 1928, Luengo & van Hall 1978, Luciano, Hauser & Benda 1983, Dargenio et al 1986). A recent animal study found no significant effect on peritoneal fibrinolytic activity but a high incidence of pulmonary effusions and perioperative deaths with intraperitoneal Dextran 70 (Wagaman et al 1986). Pleural effusions have also been reported after the use of Dextran in human beings (Adoni et al 1980). Two clinical studies with second-look laparoscopy have evaluated the effect of Dextran in women with pelvic adhesions (Rosenberg & Board 1983, Adhesion Study Group 1983). Both showed a decrease in subjective adhesion score but Dextran did not prevent the reformation of adhesions after surgical division.

Non-specific proteolytic enzymes have been used to digest proteins, including fibrin, in the inflammatory exudate. Pepsin and trypsin are both rapidly neutralised by the peritoneal exudate and were shown to be ineffective as long ago as 1922 (Kubota). Papain, derived from the papaw, is another such enzyme which has been widely studied. Initial promising results (Ochsner & Mason 1928) were not substantiated by others (Lehman & Boys 1940).

More recently oral papain given to monkeys reduced the severity, but not incidence, of adhesions (Kapur, Talwar & Gulati 1969a) and intraperitoneal papain had no effect on adhesion formation in rats (Stevens 1968).

1.5.3 Separation of peritoneal surfaces

Inert polysiloxanes have been used to separate adjacent peritoneal surfaces (Cook 1964). Studies in dogs, (Malette & Eiseman 1965, Punnoose & Sachdeva 1968) and rabbits (Aboulafia & Polishuk 1967) gave encouraging results but Furman & Denize (1966) found no benefit in rats after mechanical peritoneal injury. Also adhesions in silicone treated rats were denser with granuloma formation compared to controls (Brody & Frey 1968). Oxidised cellulose has similarly been found to be ineffective, either alone or in combination with Dextran 70 (Schroder et al 1982).

Polyvinylpyrrolidone is a synthetic polymer of molecular weight 12.6 kD which has been claimed to act as a barrier to adhesion formation (Mazuji, Kalambaheti & Pawar 1964, Goldberg, Sheets & Habal 1980). These reports, though, were contradicted by the findings of Hugh & Ellis (1964) where the incidence of adhesions was actually increased by this agent.

As well as these agents a number of other ingenious methods have been used to separate bowel surfaces. These include oxygen distension of the peritoneal cavity

(Bainbridge 1909), olive oil (Claypool et al 1910), ox peritoneum (Foster 1930), liquid paraffin (Norris & Davison 1934), amniotic membrane (Kubanyi 1947), chyme (Armbruster & Khawaja 1964), Surgicell (Larsson, Nisell & Granberg 1978) and Gore-tex membrane (Goldberg, Toledo & Mitchell 1987). None has proved useful.

An alternative approach has been artificially to stimulate bowel peristalsis to prevent adjacent loops from adhering. One of the most novel suggestions was that the bowel should be filled with iron filings and a magnet passed over the abdominal wall (Payr 1913)! Both prostigmine (Cone 1959) and cisapride (Sparnon & Spitz 1989) have been advocated but only limited reduction in adhesion formation was reported.

1.5.4 Inhibition of fibroblastic proliferation

Several approaches have been taken to diminish the inflammatory reaction to peritoneal injury and to inhibit fibroplasia resulting in decreased organisation of fibrin. The incidence and density of adhesions to sites of mechanical injury in the rat were reduced by systemic administration of the antihistamine, methapyrilene (Jacqmain & Shumacker 1962). However the protease inhibitor, aprotinin, was not found to affect adhesion formation (Raftery 1979b). Cortisone is known to reduce the formation of granulation tissue and inhibit

fibroblastic proliferation. Eskeland (1963) showed reduced adhesion formation in rats given intraperitoneal prednisolone provided it was delivered within 48 hours of injury. This effect of steroids has been substantiated by others (De Sanctis, Schatten & Weckesser 1955, Shikata & Yamaoka 1977, Roch 1978). Increasing doses of steroids resulted in abscess formation despite the mechanism of injury being non-infective. When an anastomosis was fashioned in the adhesion model peritonitis and death occurred in 75% of animals treated with prednisolone (Levy & Ducasse 1965). Conversely Glucksman & Warren (1966) were unable to show any benefit of steroids in preventing the reformation of adhesions after surgical lysis in dogs.

One intraperitoneal dose of hydrocortisone has been evaluated in women undergoing surgery for ectopic pregnancy (Swolin 1967). All patients underwent laparoscopy at three months and the treated group had significantly fewer adhesions. However, surgical techniques were not standardised between the two groups making interpretation of results difficult. Combination of steroids with anti-histamines has also been suggested to be beneficial (Replogle, Johnson & Gross 1966). Large systemic doses were required to reduce adhesion formation in dogs and the studies were extended to human beings but with insufficient data for assessment.

It appears that steroids must be given in high doses to

exert an anti-adhesion effect and this is associated with an excessive morbidity (Grosfield et al 1973). Similarly the use of cytotoxic agents to inhibit fibroplasia results in unacceptable side effects. There was complete abolition of adhesion formation in rats receiving fluorouracil but the dose required resulted in a mortality in excess of 50% (Goldman & Rosemond 1967).

Non-steroidal anti-inflammatory drugs have also been evaluated in a number of studies. Oxyphenbutazone has been reported to inhibit adhesion formation in two animal models (Kapur, Talwar & Gulati 1969b, Larsson, Svanberg & Swolin 1977). Ibuprofen has been more widely studied with variable results; benefit being demonstrated in some studies (Bateman, Nunley & Kitchin 1982, Nishimura, Shimanuki & diZerega 1984) and no effect in others (Holtz 1982, Luciano, Hauser & Benda 1983).

Progesterones are known to have anti-inflammatory properties including reducing vascular permeability and inhibiting granulation tissue but not affecting collagen breakdown (Nakagawa et al 1979). Intraperitoneal progesterone reduced adhesion formation in a guinea-pig model (Maurer & Bonaventura 1983) and systemic administration was effective in a rabbit model (Nayel et al 1988). Conversely adhesion formation was actually increased by local progesterone in a further study (Holtz et al 1983).

Other agents which are claimed to reduce adhesion formation include colchicine (Shapiro, Granat & Sharf 1982, Granat et al 1983) and Vitamin E (Kagoma et al 1985) but these reports remain unsubstantiated.

1.5.5 Controlled adhesion formation

With no acceptable effective method for eliminating adhesions by pharmacological means, surgeons have devised a number of methods for controlling the almost inevitable formation of adhesions after abdominal surgery. The rationale is that adhesions forming in a controlled manner without "kinks" in the bowel will not lead to complications.

Plication of the intestine was popularised by Noble (1937). In 260 patients treated by this technique there was a mortality of 1% and no recurrence of obstruction over a follow up of one to twenty years (Noble 1950). Connolly & Smith (1960), combining several series, reported an average obstruction rate of 12% in patients treated with this method. Transmesenteric plication has been proposed as a safer alternative (Childs & Phillips 1960) but neither technique completely prevents further obstructive episodes and both may result in serious complications (McCarthy 1975, Somell 1978).

Another method for controlled adhesion formation is by threading a long tube down the whole length of the small

intestine which, by its intrinsic stiffness, prevents the bowel kinking while adhesions develop (Baker 1959). In a recent study no obstructive episodes occurred in 32 patients treated this way over a 1-5 year follow up (Munro & Jones 1978).

1.5.6 Conclusions

It is clear that none of the above methods which have been advocated is ideal. Each new agent or method, after an initial period of enthusiasm, has been largely abandoned. The absence of any universally employed method of adhesion prevention in current surgical practice is testimony to the failure of these proposed methods. Our knowledge of peritoneal healing and the pathophysiological events in adhesion formation indicate that the presence of fibrin, and its subsequent organisation, is central to the adhesive process. Fibrin is present in a peritoneal wound for up to five days (Eskeland 1966a) and fibroblast invasion with collagen deposition starts at three days (Milligan & Raftery 1974). However adhesions do not develop after all peritoneal insults. There thus appears to be a balance between the removal of fibrin and the organisation of the fibrinous exudate (Figure 1.2). In other systems the fibrinolytic pathway is responsible for the removal of fibrin and there is strong evidence that the peritoneum is also capable of fibrinolysis.

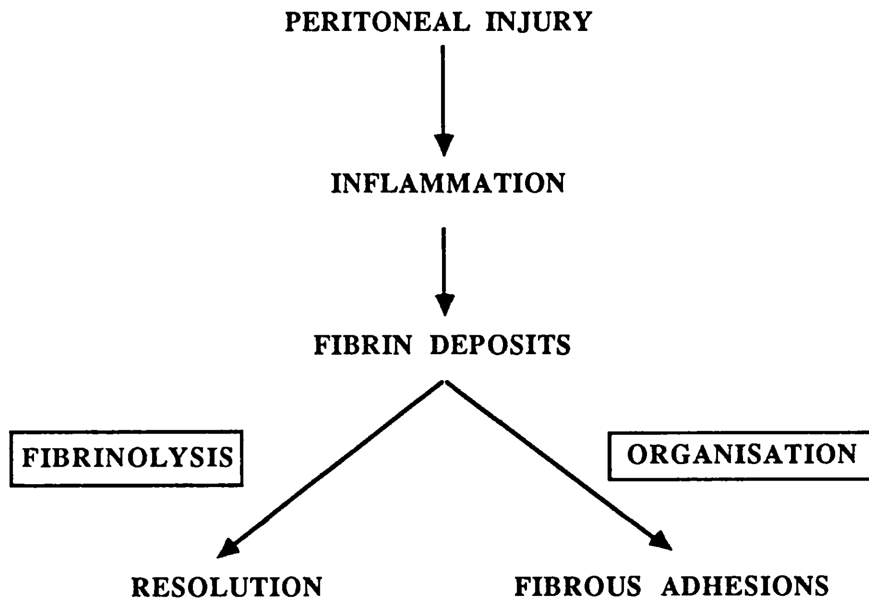

Figure 1.2 *Adhesion formation is a balance between the degradation and organisation of fibrin*

1.6 PERITONEAL FIBRINOLYSIS

Hartwell (1955) postulated that mesothelial cells prevented adhesions by combining their ability for healing rapidly as a single sheet of cells with fibrinolytic properties. At that time, though, there was no evidence that mesothelial cells possessed fibrinolytic activity. However, in 1969, Myhre-Jensen, Larsen & Astrup embedded histological sections of rat, guinea-pig and rabbit peritoneum in plasminogen-containing fibrin on microscope slides and incubated the specimens at 37°C. After 10 to 50 mins incubation, discrete areas of lysis could be localised to the mesothelial surface. No lysis was detected in fibrin slides that had been heated to 80°C. At this temperature the plasminogen present on the slide is denatured and any lysis of fibrin that occurred would be due to non-specific proteolysis. It was concluded that the fibrinolysis observed with mesothelium was due to the presence of activators of plasminogen in the mesothelial tissue. Activity could not however be detected in rabbit peritoneum even after prolonged incubation

The same phenomenon of peritoneal fibrinolysis was produced by 5 mm^2 biopsies of dog peritoneum placed directly on plasminogen-containing fibrin plates (Porter et al 1969). Sections of human peritoneum placed on fibrin slides indicated the presence of plasminogen activators in the submesothelial blood vessels after 5 min incubation and

along the mesothelial border after 20 min incubation (Porter, Ball & Silver 1971).

1.6.1 Reduction of peritoneal fibrinolytic activity

In the studies of Porter, Ball & Silver (1971) mechanical abrasion of human peritoneum *in vitro* and subsequent incubation on fibrin slides resulted in a 38% reduction in fibrinolysis compared with controls. Thus, fibrinolytic activity of human peritoneum was shown to be reduced by mechanical trauma as may occur during operative procedures.

Mechanical abrasion injury was performed *in vivo* to an 18 inch length of dog ileum (Gervin, Puckett & Silver 1973). This resulted in diminished fibrinolysis compared with controls, and 14 of 15 (93%) animals, in whom fibrinolytic activity was reduced by greater than 50%, formed adhesions. Caecal abrasion injury in rats (Buckman et al 1976a) also reduced fibrinolytic activity; maximally at 24 hours and returning to normal by 72 hours. This time period overlaps with the stage at which fibrinous adhesions are known to become organised by fibroblast proliferation (Milligan & Raftery 1974). In the same studies the suturing of a 2 cm^2 free (and therefore ischaemic) peritoneal graft resulted in a 65-80% reduction in fibrinolytic activity and was associated with adhesion formation in all animals. Similarly, the production of a

fibrinopurulent peritonitis from a standardised model of gangrenous small bowel resulted in complete abolition of fibrinolytic activity in the parietal peritoneum of dogs and adhesion formation in all animals (Hau, Payne & Simmons 1979).

1.6.2 Site of peritoneal fibrinolysis

These studies all showed reduction in fibrinolytic activity of peritoneal biopsies. However the precise site of production of the plasminogen activators had not been studied. In an attempt to answer this question Raftery (1979) used Häutchen preparations of 2 cm^2 rat parietal peritoneal wounds on fibrin slides. This looked at the *en face* fibrinolytic activity of the surface cells alone. In the first three days, when inflammatory cells only were present, there was no fibrinolytic activity. However, as mesothelial regeneration occurred, fibrinolytic activity was seen so that by eight days, when mesothelial regeneration was complete, a wide band of fibrinolysis was visible along the whole mesothelial border.

Merlo et al (1980) applied discs of dry gelatin to human peritoneum. The discs picked up only the surface layer of mesothelium and this was confirmed microscopically. Incubation of these discs on fibrin plates resulted in lysis for all peritoneal samples though there was some site to site variation. Gel discs not

covered with mesothelial cells had no fibrinolytic activity. In this study, fibrinolytic activity was attributed exclusively to the capacity of the mesothelial cell to produce plasminogen activators. This gel disc method of assessing fibrinolytic activity in a single layer of cells was also employed by Raftery (1981) following suturing of a free (ischaemic) peritoneal graft to a rat parietal peritoneal defect. Fibrinolytic activity was significantly reduced immediately after grafting and at 24 hours; all animals formed fibrous adhesions to the graft by two weeks.

1.6.3 Summary

The above studies, all performed in the last 20 years, provide a strong body of evidence that fibrinolytic activity, in the form of plasminogen activators, resides in visceral and parietal peritoneum. This activity is reduced following mechanical injury, ischaemia or bacterial peritonitis - all conditions associated with adhesion formation. From this work an attempt has been made to establish a unifying pathophysiological mechanism for adhesion formation. It is argued that the fibrinolytic activity of definitive mesothelial cells is able to lyse fibrinous adhesions (Raftery 1984). If the mesothelium is damaged locally, fibrinolytic activity is reduced and the balance is shifted in favour of organisation of the

fibrinous adhesions to produce permanent fibrous adhesions (Figure 1.2).

1.7 THE FIBRINOLYTIC SYSTEM

1.7.1 Historical aspects

Hunter (1794) observed that blood taken from patients who had died suddenly lost its ability to coagulate. Similarly Dastre (1893) observed this phenomenon in dogs after severe bleeding and described these findings under the title *"La Fibrinolyse"* from which the term fibrinolysis originated. Fibrinolytic activity of animal tissue was first demonstrated by Fleischer & Loeb (1915) who observed a lytic effect of samples of rat and guinea-pig tissue on clotted blood. Further studies showed that *in vitro* treatment of blood with chloroform (Dale & Walpole 1916, Tagnon 1942) or with culture fluid from strains of haemolytic streptococci (Tillett & Garner 1933) also prevented blood from clotting.

In 1941 Milstone demonstrated that the fibrinolytic effect of the streptococcal substance depended upon an accessory lytic factor in the blood which was converted to an active enzyme by the bacterial fluid or by chloroform treatment. This lytic factor is now called plasminogen,

the active enzyme plasmin and the streptococcal substance streptokinase. Chloroform was shown to act by removing an inhibitor of plasmin, now termed antiplasmin, from the blood (Christensen & MacLeod 1945).

In 1947 Astrup & Permin showed that animal tissues activated an agent which in turn activated plasminogen and named this substance fibrinokinase, though it is now known as tissue plasminogen activator (tPA). Macfarlane & Pilling (1947) found fibrinolytic activity in urine and Williams (1951) demonstrated that this was due to a different plasminogen activator now termed urokinase plasminogen activator (uPA).

The fibrinolytic system has been studied in great detail and is now recognised to be a complex system of pro-enzymes, enzymes and inhibitors. The active fibrinolytic enzyme, plasmin, is inhibited by α_2-antiplasmin (Collen 1976) which complexes with plasmin to prevent indiscriminate fibrinolysis. Similarly, inhibitors of the plasminogen activators tPA and uPA have been identified (Sprengers & Kluft 1987). The interaction of these plasminogen activators and inhibitors in the degradation of fibrin is shown in Figure 1.3.

1.7.2 Tissue plasminogen activator (tPA)

Human tPA was first obtained in pure form from uterine tissue (Rijken et al 1979) and found to be immunologically

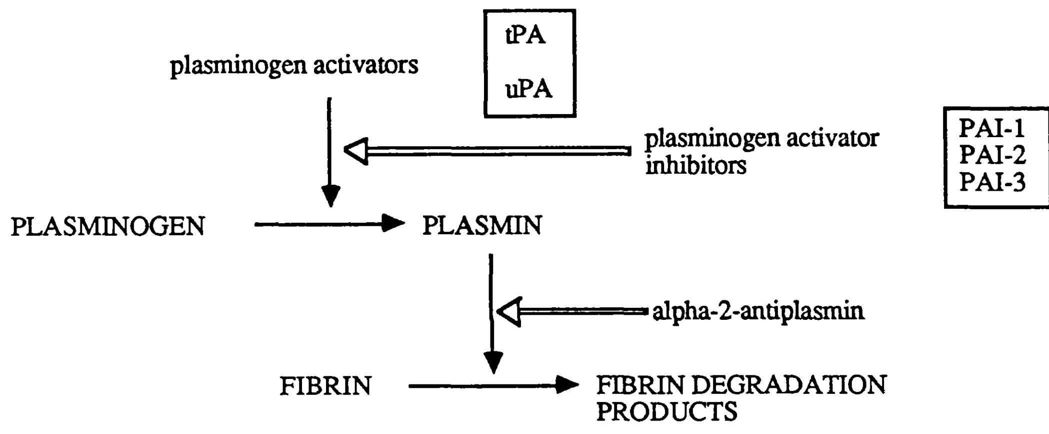

Figure 1.3 *The fibrinolytic system*

identical to the plasminogen activator released by endothelial cells and found in blood (Kristensen et al 1984, Kjaeldgaard, Larsson & Åstedt 1984). Subsequently tPA has been purified from a stable human melanoma cell line (Bowes: RPMI-7272; Collen et al 1982) and identified in a number of tissues including skin, lung, thyroid and heart (Rijken, Wijngaards & Welbergen 1981).

Natural tPA is a serine protease with a molecular weight of ≈ 70 kD existing as a single polypeptide chain (sc-tPA). On its own tPA is a poor activator of plasminogen to plasmin but this reaction is markedly potentiated (200 to 400 fold) by the presence of fibrin (Rånby 1982, Norrman, Wallén & Rånby 1985). Kinetic data support a mechanism in which fibrin provides a surface to which tPA and plasminogen adsorb in a sequential and ordered way to form a ternary complex (Hoylaerts et al 1982). The binding of sc-tPA to the active site of plasminogen results in an initial slow production of plasmin. The plasmin so generated then acts to convert sc-tPA to a two-chain form of tPA (tc-tPA) which proceeds to generate plasmin from plasminogen in a second fast phase. The high affinity of tPA for plasminogen in the presence of fibrin allows efficient conversion to plasmin on the fibrin deposit whilst preventing any appreciable activation of free plasminogen in plasma.

1.7.3 Urokinase plasminogen activator (uPA)

This activator was originally detected in human urine (Macfarlane & Pilling 1947) but has since been found in a variety of tissues including kidney, lung, placenta and bladder (Larrson et al 1982). uPA, also a serine protease of molecular weight ≈ 55 kD, is synthesised as a single chain pro-enzyme (sc-uPA) which is converted to an active two chain form (tc-uPA) by plasmin (Ichinose, Fujikawa & Suyama 1984). sc-uPA initiates the conversion of plasminogen to plasmin and the reaction proceeds more rapidly as tc-uPA is generated in a similar way to tPA (see above).

However, in contrast to tPA, uPA-mediated generation of plasmin is not influenced by the presence of fibrin and the overall reaction rate is much slower. This difference in the plasminogen activating activity of tPA and uPA has led to the proposal that tPA is the principal fibrinolytic enzyme while uPA plays a role in extravascular physiological processes, principally tissue degradation and invasion (Dano et al 1985). Whilst this separation of roles is attractive, available evidence suggests that the situation is more complex. uPA is present in low amounts in plasma and does contribute to intravascular thrombolysis. It has been demonstrated that sc-uPA is present in plasma as a complex with a competitive inhibitor and that the presence of fibrin leads to dissolution of

this complex (Collen et al 1986). Thus uPA may be synergistic with tPA, acting as a "back-up" mechanism, leading to efficient fibrinolysis.

1.7.4 Extrinsic plasminogen activation

There are also two other mechanisms of plasminogen activation. Streptokinase is produced by Lancefield Group C β-haemolytic streptococci (Brodgen, Speight & Avery 1973) and complexes with plasminogen to generate plasmin. Although in use clinically it is not a natural intrinsic human or animal physiological agent. Also factor XII (Hageman factor) can cause some intrinsic activation of plasminogen but this appears to be related to activation of sc-uPA and is not a major contribution to fibrinolysis (Ogsten & Bennet 1978, Kluft, Wijngaards & Jies 1984).

1.7.5 Plasminogen activator inhibitors

The fibrinolytic system is regulated by the recently discovered plasminogen activator inhibitors (PAI's). The occurrence of a specific PAI in plasma was confirmed in 1983 (Chmielewska, Rånby & Wiman) and it was subsequently also found in the conditioned media of endothelial cells (Loskutoff 1983). This inhibitor, named PAI-1, is a 52 kD single chain glycoprotein which is the primary inhibitor of both tPA and uPA in plasma forming an inactive 1:1 stoichometric complex with both plasminogen activators.

Studies of endothelial cells in culture have shown that PAI-1 can exist in both active and inactive forms depending on the conformation of the molecule (Hekman & Loskutoff 1985).

Platelets account for approximately 90% of circulating PAI-1 (Kruithof et al 1986). Plasma PAI-1 is present almost totally in the active form whilst PAI-1 within platelets is primarily inactive (Kruithof 1988a). Platelets thus contain a large pool of latent PAI-1 which is thought not to contribute to plasma levels under normal physiological conditions but may be important in preventing premature lysis within a thrombus (Paques 1988).

PAI-2 was initially purified from human placenta (Astedt et al 1985) and is also found in pregnancy plasma but not normal plasma (Lecander & Åstedt 1986). It has a molecular weight of 48 kD and complexes principally with uPA and, to a lesser extent, tPA (Kruithof 1988b). Its physiological function is not yet established but available evidence suggests a role in the regulation of fibrinolysis at parturition (Chmiewelska 1988).

PAI-3 was first isolated from human urine (Stump, Thienpont & Collen 1986) where it is found in complex with uPA. It is found in low concentrations in plasma and recent data suggests that PAI-3 is identical to the

inhibitor of activated protein C (Heeb et al 1987). Two chain uPA and both forms of tPA are inhibited by PAI-3 but at very slow rates (Stump, Thienpont & Collen 1986).

Protease nexin was first identified in the conditioned media of fibroblasts (Baker et al 1980) and has a broad spectrum of action. It is the most rapid inhibitor of thrombin but also inhibits trypsin, factor Xa, uPA and tPA. The precise physiological role of protease nexin and PAI-3 has not yet been established.

1.7.6 α_2-antiplasmin

α_2-antiplasmin is the most important physiological inhibitor of plasmin and was first isolated in 1976 (Collen 1976). It is a one chain glycoprotein with a molecular weight of ≈ 67 kD. α_2-antiplasmin binds to the active site of plasmin to form an inactive 1:1 stoichometric complex. This reaction occurs in two stages: a very rapid reversible first step and a slower irreversible second step. α_2-antiplasmin can also exert an antifibrinolytic effect directly on fibrin deposits, because it can be cross-linked to the fibrin surface by factor XIIa (Sakata & Aoki 1982) preventing premature lysis of fibrin clots. However, plasmin generated by plasminogen in a ternary complex with tPA and fibrin is protected from α_2-antiplasmin as its binding site is already occupied. The main role of

α_2-antiplasmin is to inactivate free circulating plasmin thus preventing indiscriminate systemic fibrinolysis.

Whilst understanding of the regulation of fibrinolysis by specific inhibitors is incomplete (Sprengers & Kluft 1987), current knowledge indicates that PAI-1 is the principal physiological inhibitor of tPA and α_2-antiplasmin of plasmin.

1.7.7 Physiological fibrinolysis

The fibrinolytic system, in combination with the coagulation cascade, plays an integral role in the maintenance of vascular haemostasis. Fibrin is formed at the site of any vascular injury and the fibrinolytic enzymes, as part of a dynamic system, play a central role in preventing thrombosis and maintaining vascular integrity. Plasminogen and tPA adsorb to the fibrin clot in an ordered and sequential manner. This binding leads to conformational changes in both molecules which facilitates efficient activation of plasminogen to plasmin. The plasmin formed on the fibrin surface has its active site occupied and is thus only slowly inactivated by α_2-antiplasmin. In plasma minimal plasminogen activation by tPA occurs and α_2-antiplasmin rapidly binds to free plasmin thus preventing fibrinogen degradation. By these mechanisms the normal fibrinolytic process is both

triggered by and localised to the sites of fibrin formation (Collen & Lijnen 1985).

The vascular endothelium is the site of continuous release of tPA into the circulation (Kjaeldgaard, Larsson & Astedt 1984). A number of physical and chemical stimuli are known to increase tPA plasma levels but the precise mechanism is uncertain (Rånby & Brandstrom 1988). The short half-life of tPA (5 mins) and the rapid complexing of PAI-1 with tPA prevents indiscriminate plasminogen activation. The rapid reappearance of plasma PAI-1 after infusion of excess tPA indicates a rapid equilibrium between release and clearance (Emeiss 1986).

Plasminogen activators have also been shown to play an important role in a number of other systems. They are secreted by granulosa cells to degrade the follicle wall just prior to ovulation (Beers, Strickland & Reich 1975) and play a part in embryo implantation and development (Strickland, Reich & Sherman 1976). Mammary gland involution after lactation represents a rapid degradation of several tissue components and closely correlates with the production of plasminogen activators by mammary epithelial cells (Ossowski, Biegel & Rich 1979). There is also growing evidence to connect plasmin with prohormone processing. Plasmin converts the prohormone lactogen to the more active form (Russell et al 1979) and may

participate in insulin metabolism (Geiger & Binder 1984). Cells of the anterior and intermediate pituitary lobes secrete a mixture of uPA and tPA and these are postulated to play a part in hormone metabolism (Kristensen et al 1985).

The ability of cancer cells to penetrate structures between various tissue and cell types has been related to increased plasminogen activator activity of malignant cells *in vitro* and *in vivo* (Markus 1988). These activators are able to degrade the glycoproteins laminin and fibronectin in basement membranes and the extracellular matrix (Liotta et al 1981, Bogenmann & Jones 1983). Plasminogen activator synthesis has been directly linked to neoplasia with a rapid onset of production in cells transformed by oncogenic viruses or chemicals (Unkeless et al 1974, Sisskin et al 1980). In general, *in vivo* tumours seem to contain more plasminogen activating activity than the corresponding normal tissue (Corasanti et al 1980, Dano et al 1985). In experimental systems a positive correlation has been found between plasminogen activator production and tumourogenicity as well as other parameters of the transformed state (Newcomb, Silverstein & Silagi 1978, McCabe & Evans 1982). Also, antibodies which inhibit human uPA have been shown to interfere with the metastatic propensity of a human cell line transplanted into chick embryos (Ossowski & Reich 1983).

These findings all suggest that the fibrinolytic system, and in particular plasminogen activators, play specific pathophysiological roles in a number of tissues and systems. Current knowledge of the precise role and regulation of these processes is expanding rapidly.

1.8 STUDY AIMS

The aim of the studies described in this thesis was to develop further understanding of the relationship between the fibrinolytic system, peritoneal physiology and adhesion formation. More specifically to develop a reproducible assay for measuring fibrinolytic activity of animal peritoneum and to relate the peritoneal fibrinolytic response to standard insults with regard to the timing of these events. In addition it was proposed to examine in detail the changes in peritoneal fibrinolysis in normal and inflamed human peritoneum and elucidate the underlying molecular mechanisms for these changes. On the basis of these findings it was hoped that it would be possible to develop a strategy for the prevention of intra-abdominal adhesions and to test this in an animal model.

CHAPTER 2

MEASUREMENT OF PERITONEAL FIBRINOLYTIC ACTIVITY

2.1 Introduction

Fibrinolytic activity of animal tissue was first demonstrated by Fleisher & Loeb (1915) who observed the lytic effect of samples of rat and guinea-pig tissue incubated with clotted blood. In 1947 Astrup & Permin produced fibrin plates from a mixture of bovine fibrinogen and thrombin and observed lysis when either animal or human tissue was placed on the plates. They named this tissue activator of fibrinolysis, fibrinokinase. Whilst their experiment showed the presence of tissue fibrinolysis, it did not determine whether the factor in the tissue was plasmin itself, acting directly on the fibrin plate, or an activator of plasminogen to plasmin. In 1952 Lassen showed that tissues did not produce lysis on fibrin plates that had prior heat treatment to 80°C which destroyed any plasminogen present. Lysis did occur on unheated plates indicating that tissue fibrinolysis was mediated through a plasminogen activator (PA).

Initial attempts at extraction of this tissue PA proved difficult due to its low solubility in water or saline. Extraction with potassium thiocyanate produced an improved yield (Astrup & Stage 1952). Subsequently it has been shown that the extraction of PA from tissue can be performed with acetate (Bachmann et al 1964) and also with the detergent Triton X-100 (Ali & Lack 1965).

Quantification of the fibrin plate assay became

possible with the establishment of standard solutions of PA derived from pig heart (Astrup & Albrechtsen 1957) and subsequently standard solutions of urokinase (Kok & Astrup 1969). The development of techniques allowing the production of recombinant tissue plasminogen activator (rtPA) has now enabled this agent to be used for quantification to give a standard measure of plasminogen activating activity (PAA).

Todd (1958) described a method for the histological localisation of the plasminogen activator. A section of the tissue in question was embedded in a thin film of fibrin on a microscope slide and incubated at 37°C. After fixation and staining, fibrinolytic activity was detected by an area of lysis which could be related to specific cellular components of the tissue in question. This method was used to localise PAA to venous endothelium (Todd 1959). The fibrin slide technique is useful for determining the cellular site of PAA but reliable quantification is not possible.

Assay of PAA may be performed in a variety of tissue preparations - whole tissue, tissue extracts and, more recently, a single layer of cells obtained by adhering a surface layer of cells to gelatin discs (Merlo et al 1980). Ideally an assay for PAA should measure all avilable PAA in the tissue in question, be quantifiable and allow comparison between samples.

In the present study samples of peritoneum were assayed quantitatively using a modified fibrin plate method to determine the most appropriate animal for study of peritoneal PAA and the most suitable tissue preparation.

2.2 Materials and Methods

2.2.1 Animal studies

Adult male Wistar rats 250-300 g (n=6; Harlan UK, Ltd), male New Zealand White rabbits 2-2.5 kg (n=6; Regal Rabbits, UK) and male Dunkin-Hartley guinea-pigs 600-800 g (n=6; Porcellus Animal Breeding Ltd., UK) were studied. Under general anaesthesia (inhaled ether/air mixture for rat and guinea-pig and intravenous 6% w/v sodium pentobarbitone (Sagital, May & Baker, UK) delivered by the marginal ear vein for rabbit) a midline laparotomy was performed. Peritoneal biopsies and geldisc mesothelial imprints were taken from the parietal peritoneum, at least 2 cm from the edge of the midline wound, of each animal as soon as possible after opening the abdomen.

2.2.2 Punch biopsy technique

Peritoneal biopsies were taken using a disposable, 6 mm diameter biopsy punch (Stiefel Laboratories (UK) Ltd., Wooburn Green, UK). After the peritoneum had been incised

with the biopsy punch, the disc of peritoneum was carefully dissected off the underlying tissue, wrapped in aluminium foil and frozen in dry ice before transfer to a freezer at -80°C.

2.2.3 Geldisc biopsy technique

Sterile gelfilm (Upjohn Ltd., USA) kept moist in isotonic saline was cut into discs using a 6 mm biopsy punch. The discs were allowed to dry and after opening the peritoneal cavity applied to the parietal peritoneum for 10 seconds. The disc was then placed on blotting paper with the peritoneal-sample surface uppermost. The discs were assayed immediately by placing them on a previously prepared fibin plate (*vide infra*).

2.2.4 Extraction of plasminogen activating activity from punch peritoneal biopsies

Peritoneal punch biopsies were thawed rapidly at room temperature and weighed. Each biopsy was washed with 0.5ml rinsing solution (5 mM sodium dihydrogen phosphate, 0.15M sodium chloride; pH 7.4) and placed into 1 ml homogenising solution (2.5 mM sodium dihydrogen phosphate, 0.075 M sodium chloride, 0.25% Triton X-100 (Sigma Chemical Co. Ltd., UK); pH 7.8) in a small plastic tube on ice. The tissues were then homogenised using an Ultra-Turrax homogenizer (Janke and Kunzel, FRG) for 30 s. The

homogenates were centrifuged at 12 000 x *g* for 20 min at 4°C. Aliquots of 0.25 ml of supernatant were stored at -20°C until assay. All assays were performed within four weeks.

2.2.5 Preparation of fibrin plate

A layer of fibrin was produced in the base of 5 cm diameter sterile Petri dishes (Sterilin Ltd., UK) by pouring in a solution containing 0.9ml Veronal buffer (0.03 M sodium acetate, 0.03 M sodium barbitone, 0.02 M hydrochloric acid, 0.7% sodium cloride; pH 7.4), 3 ml of 1% human fibrinogen (KabiVitrum Ltd., Stockholm, Sweden), 0.3ml human plasminogen (KabiVitrum Ltd) and 0.2ml bovine thrombin (Armour Pharmaceutical Co., USA; 20 units/ml). Once the fibrin solution had set, a 6 mm well was cut in the centre of the plates with the 6 mm biopsy punch and used for assay of the peritoneal homogenates. The gelfilm discs and whole tissue samples were assayed on fibrin plates without a central well.

2.2.6 Determination of plasminogen activating activity

To determine the PAA of the peritoneal biopsies, 20 µl of each peritoneal homogenate was placed in the well of a fibrin plate. The gelfilm disc samples were placed on an intact fibrin plate; the surface that had been in contact with the peritoneum bing placed directly onto the fibrin.

The whole peritoneal biopsies were washed with the rinsing solution described above and placed peritoneal surface down directly on fibrin plates. All assays were performed in duplicate.

Standard solutions of human recombinant tPA (rtPA, 2nd international standard for tPA, National Institute for Biological Standards and Controls, Mill Hill, UK) were reconstituted in sterile water at six concentrations ranging from 0.0425 IU/ml to 8.5 IU/ml. Aliquots (20 µl) from these standard solutions were placed directly on one series of fibrin plates and in the central well of a second series of plates.

This method was based on previous work which showed a linear relationship between the logarithm (to the base ten) of standard enzyme doses applied to fibrin plates and the lysis zone diameter produced (Haverkate & Traas 1974). The dilutions of rtPA chosen for the standard solutions in the present study were based on preliminary work which had shown that these amounts of rtPA produced a zone of lysis on fibrin plates of diameter 0.7 to 2.2 cm.

All the fibrin plates were then incubated for 24 hours at 37°C and the diameter of the zone of lysis around the standard solution and the samples measured directly by placing the plates on top of a measuring scale. The maximum and minimum diameters were recorded and the mean value used (Figure 2.1).

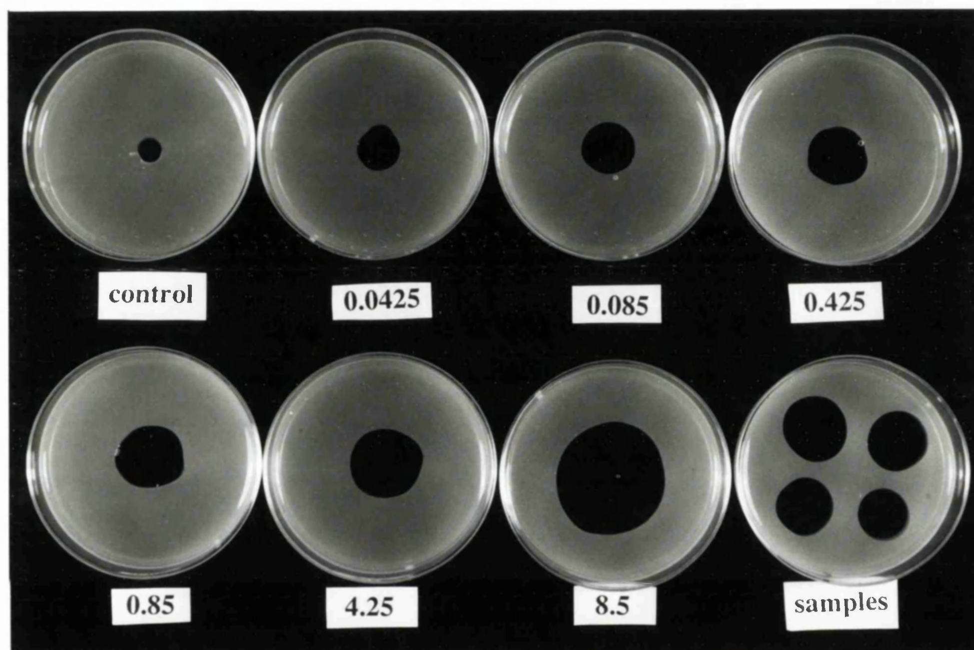

Figure 2.1 *Fibrin plate lysis zones exhibited by: homogenising buffer (control), recombinant tissue plasminogen activator standard solutions (0.0425, 0.085, 0.425, 0.85, 4.25, 8.5 IU/ml) and peritoneal biopsy homogenate (sample) after incubation at 37°C for 24 hours*

2.2.7 Control samples

Six samples from each animal species studied were incubated on fibrin plates that had been previously heated to 80°C for 20 minutes. Heating inactivates the plasminogen in the plate, thus any lysis occurring on these plates was due to direct proteolysis and not to plasminogen activation.

Additionally, "blank" geldiscs, rinsing solution and homogenising buffer alone were incubated on fibrin plates.

2.2.8 Calculation of PAA

A plot of diameter of fibrin plate lysis against $\log_{10}$ rtPA (number of IU of rtPA in each 20 µl aliquot) was constructed for the standard rtPA solutions and the line of best fit calculated using least squares regression analysis. From this standard curve (Figure 2.2) the PAA level of the sample solutions in international units was calculated: the zone of lysis for each sample solution was interpolated with the standard curve to give the coresponding $\log_{10}$ IU of rtPA and converted by antilog to give the amount of IU of rtPA in the samples. This calculation procedure was performed rapidly by using a computer program written for this purpose (Quattro, Borland Ltd, USA).

As the punch biopsy tissue was homogenised in 1 ml of solution, each 20 µl aliquot of peritoneal homogenate

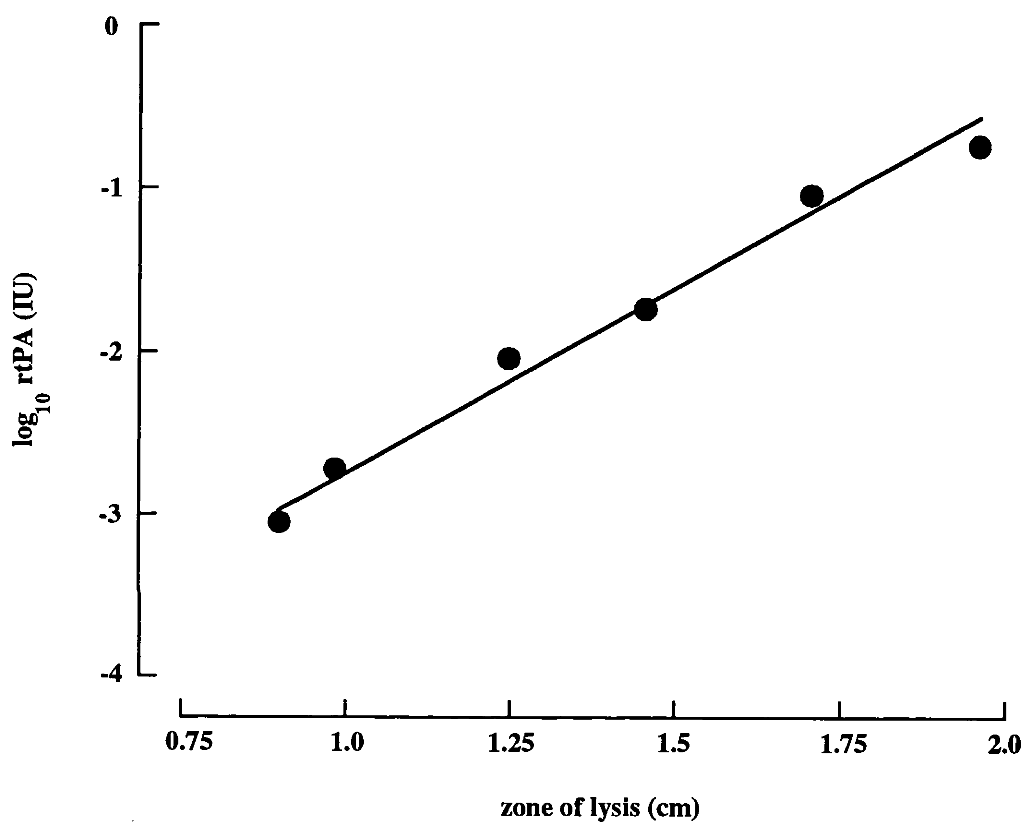

Figure 2.2 *Example of a standard curve of log_{10} rtPA (number of IU of rtPA in a 20 µl aliquot of standard rtPA solutions) versus zone of lysis of fibrin plate (n=6, r=0.99, $p<0.0001$)*

represents the activity in approximately 1/50th of the 6 mm diameter peritoneal biopsy. The area of a 6 mm diameter biopsy is 0.283 cm^2 (πr^2) and thus peritoneal PAA was expressed as IU/cm^2 by multiplying by a correction factor of 176.7.

i.e.

$$\frac{\text{activity (IU)} \times 50}{0.283 \text{ cm}^2} = \text{PAA (IU/cm}^2\text{)}$$

Using this conversion factor the lower limit of detection for the fibrin plate assay was 0.006 IU/cm^2 for the plates with central wells and 0.01 IU/cm^2 for the intact fibrin plates without wells.

2.2.9. Validation of fibrin plate assay

Standard solutions of recombinant tPA reconstituted in sterile water at five concentrations ranging from 0.085 to 8.5 IU/ml were each incubated on five fibrin plates all prepared from the same stock solution to determine the intra-assay coefficient of variation.

To determine the inter-assay coefficient of variation one sample of recombinant tPA (0.85 IU/ml) was assayed on fibrin plates prepared from five different stock solutions. The following calculation was used:

$$\text{coefficient of variation} = \frac{\text{SD}}{\text{mean}} \times 100\%$$

2.3 Results

The results obtained from the experiments described in this Chapter are outlined below and detailed in the Appendix.

2.3.1 Control samples

No lysis was observed on fibrin plates that had been heated to 80°C indicating that the fibrinolytic activity observed in all other plates was not due to plasmin in the test samples or to non-specific proteolysis but mediated via activation of plasminogen. It is subsequently referred to as plasminogen activator activity (PAA).

No lysis of unheated fibrin plates was observed with rinsing solution, homogenising solution or the geldiscs alone indicating that these did not contain plasminogen activating activity.

2.3.2 Rat PAA

Median PAA for rat peritoneum using the punch biopsy homogenate technique was 0.18 IU/cm^2 (range 0.12 to 0.23 IU/cm^2); for the geldisc samples 0.0285 IU/cm^2 (range 0.02 to 0.05 IU/cm^2); and for the whole tissue 0.011 IU/cm^2 (range $\leq$0.01 to 0.037 IU/cm^2).

Examination of the geldiscs under light microscopy showed them to contain a single layer of cells but in some

instances this was incomplete. It was not possible to examine microscopically the geldiscs which were placed on fibrin plates. Spearman rank correlation of the peritoneal PAA calculated using the punch biopsy homogenates with that measured using geldiscs showed a poor correlation (Figure 2.3; r=0.373. p=0.46).

2.3.3 Rabbit PAA

In three animals, PAA was undetectable in the peritoneal homogenates. The median PAA of normal rabbit peritoneum in homogenates from the other three animals was 0.0186 IU/cm². There was no detectable PAA in the geldisc and whole tissue samples from any of the rabbits.

2.3.4 Guinea-pig PAA

The median PAA of normal guinea-pig peritoneal homogenates was 0.102 IU/cm² (range 0.05 to 0.134). The geldisc and whole tissue samples all showed slight activity but the zones of lysis were small and irregular making quantification unreliable.

The PAA for peritoneal homogenates of all three animals is shown in Figure 2.4. Using the Mann-Whitney U test the differences between the species are significant at the 5% level.

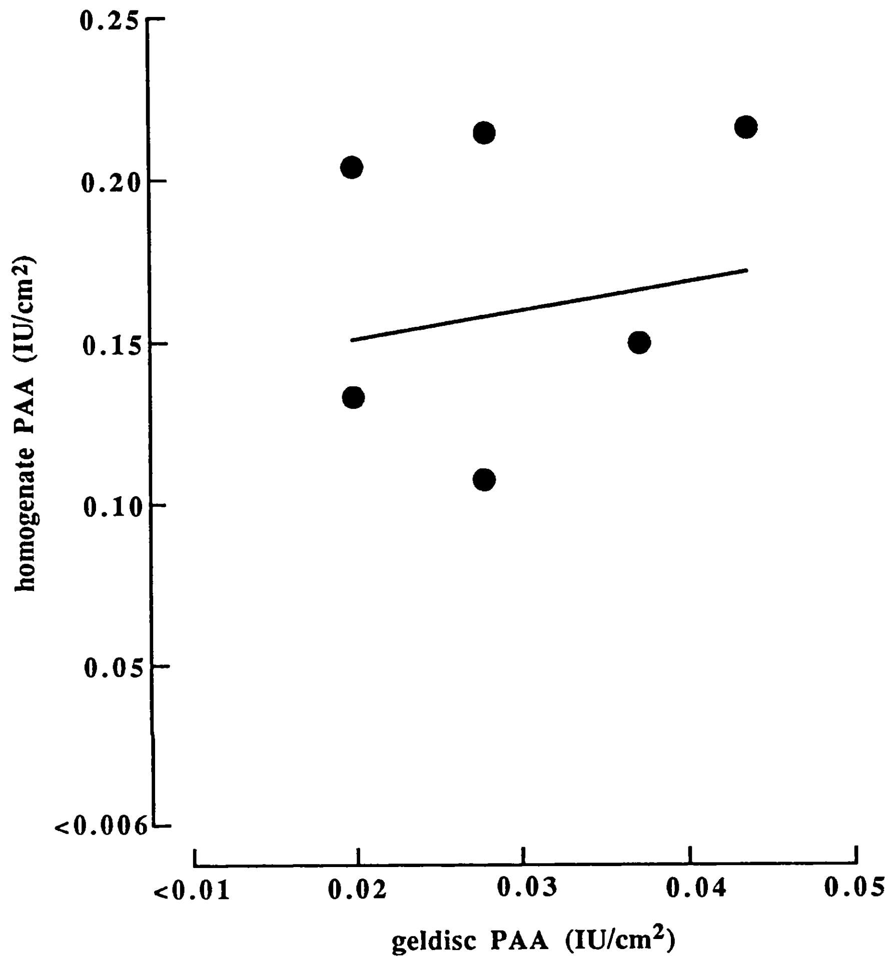

Figure 2.2 *Correlation of the plasminogen activating activity (PAA) of rat peritoneum assayed using the punch biopsy and geldisc methods ($n=6$, $r=0.373$, $p=0.46$)*

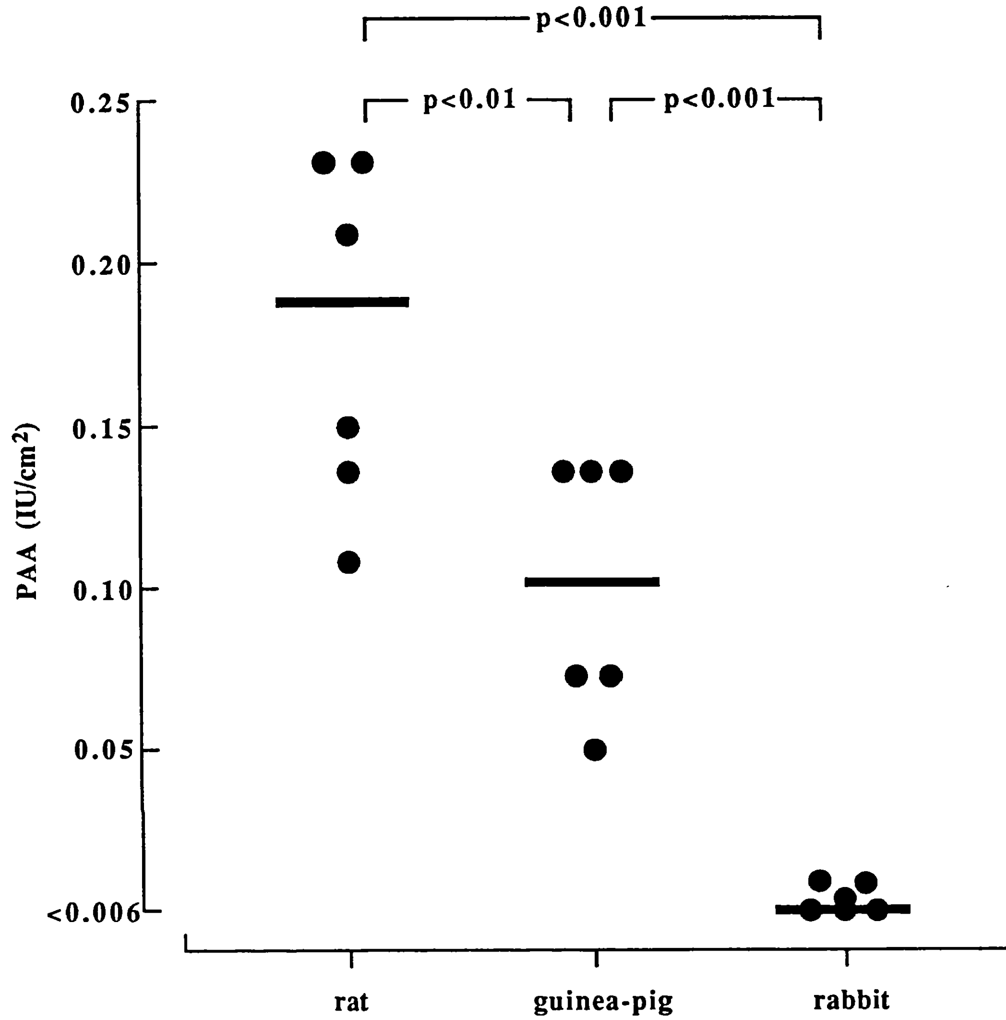

Figure 2.3 *Plasminogen activating activity (PAA) of rat, guinea-pig and rabbit peritoneum using the punch biopsy homogenate method*

2.3.5 Reproducibility of PAA measurement

For the fibrin plate method the coefficient of variation was 7.9-14.4% (intra-assay) and 6.7-13.9% (inter-assay).

Discussion

There was a statistically significant variation in the measured peritoneal PAA of the three animals studied. Myhre-Jensen, Larsen & Astrup (1969) also found fibrinolytic activity in rat and guinea-pig peritoneum but none in rabbit. In their studies the fibrin slide technique was used and thus activity was not accurately quantified. However, zones of lysis appeared more quickly around rat rather than guinea-pig peritoneum suggesting greater activity in the former. Other workers have since demonstrated PAA in rat peritoneum on fibrin plates (Buckman et al 1976a & 1976b, Raftery 1979a).

The absent or very low PAA in rabbit peritoneum may truly reflect low amounts of peritoneal PA in this animal but it would be unusual for such differences of a common enzyme to occur between similar animal species. An alternative explanation is a species-difference between rat, rabbit and guinea pig PA in their specificity for the human plasminogen used in the fibrin plate assay. The only other animals to have been studied for peritoneal PAA are

dogs (Porter et al 1969, Gervin, Puckett & Silver 1973, Hau, Payne & Simmons 1979) and cattle (Pugatch et al 1970, Trent & Bailey 1986) with activity present in the peritoneum of both species. Different methods of tissue preparation, fibrin plate composition and quantification preclude the direct comparison of results.

Investigators in these other studies have all used intact samples of peritoneum to measure PAA. Whilst this gives an acceptable result in most species tested it may not measure fully the PAA of peritoneum. It is known that PA is bound to tissue; indeed originally it was thought that PA was present in two distinct forms - a stable tissue-bound form and a labile free form (Astrup & Sterndorff 1956). These are now known to be the same form of PA and it is the extraction of PA from tissues with thiocyanate or Triton X-100 which allows measurement of full activity. The results reported in this study are the first using extraction of animal PA from a peritoneal homogenate.

A disadvantage to both the use of intact peritoneal biopsies and the homogenate method is that the precise source of the peritoneal PAA is not identified. Inevitably with a peritoneal biopsy, although the surface peritoneum is carefully dissected free, some submesothelial tissue is present. Consequently PAA detected in homogenates may be from both mesothelium and supporting tissue. This question

has been answered to some extent by incubation of animal peritoneum on fibrin slides. Myhre-Jensen, Larsen & Astrup (1969) showed that activity in rat and guinea-pig peritoneum was limited to the serosa and blood vessels. Using Häutchen preparations of rat peritoneum on fibrin slides Raftery (1979a) showed PAA confined to the mesothelial surface. The geldisc technique was initially developed to help to overcome this problem by only picking up mesothelial cells (Merlo et al 1980). As indicated in our study, this method does localise PAA to rat mesothelium so confirming the findings of Raftery (1981a). A disadvantage of this technique is the possibility of an incomplete mesothelial covering on the disc as we observed in some specimens. Unfortunately it is not possible both to assay the disc and confirm complete mesothelial cover which introduces doubt into the accuracy of quantification and to some extent invalidates comparisons between samples. Also, unlike peritoneal homogenates which can be stored for future assay, geldiscs have to be assayed immediately and it is not possible to repeat the assay to validate results.

Compared with peritoneal homogenates, relatively low levels of PAA were found with the geldisc method. This may be due to incomplete sampling by the geldisc or may reflect additional PAA associated with other structures in the peritoneal homogenate. As tissue PA is bound by cells, the geldisc method will only measure free PA associated with

the surface mesothelial cells. The homogenate technique is more able to extract bound PA and and thus give an apparently higher measure. These assay differences need to be taken into account when studying injured peritoneum. The geldisc may not measure "surface PAA" when the mesothelium is damaged whilst a homogenate of damaged peritoneum will give a measure of the PAA associated with such an area of peritoneum.

It is also important to consider exactly what is measured by the fibrin plate technique. Absence of lysis on heated plates confirms that rat and guinea-pig peritoneum mediate fibrinolysis through plasminogen activation. However, there may also be inhibitors of PA present in the peritoneal extracts and thus the zone of lysis obtained does not measure the absolute amount of plasminogen activator but only the biologically active proportion. In effect the fibrin plate method used in this study gives a measure of the functional fibrinolytic activity found in the peritoneal samples and expressed as PAA (Figure 2.5).

The studies described in this chapter indicate that all three animals studied possess functional fibrinolytic activity in normal peritoneum, with the assay more sensitive to rat PAA.

Because there may be slight variation in stock solutions when making batches of fibrin plates, a standard

curve has to be constructed for each assay run. However the intra-assay and inter-assay coefficients of variation show that the fibrin plate technique is a valid method of comparison between samples.

On the basis of the results described, the rat was chosen for further animal studies and peritoneal functional fibrinolytic activity was quantified using punch biopsy homogenates measured on a fibrin plate.

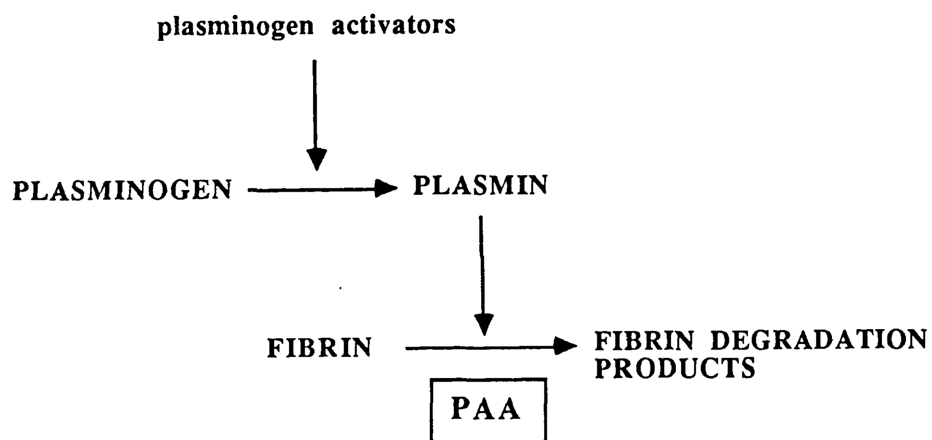

Figure 2.5 *Plasminogen activating activity (PAA) is a functional measure of the degradation of fibrin and represents a measure of biologically active plasminogen activators*

CHAPTER 3

FIBRINOLYTIC ACTIVITY OF HUMAN PERITONEUM

3.1 Introduction

The acute inflammatory exudate which follows peritoneal injury results in fibrinous adhesions (Jackson 1958). As discussed in Chapter 1 it has been suggested that the inherent fibrinolytic activity of normal peritoneum is one of the factors that determines whether these fibrinous adhesions are removed before they become organised into permanent fibrous adhesions (Buckman et al 1976a, Raftery 1981b). Peritoneal fibrinolytic activity in human beings has been demonstrated using the geldisc technique (Merlo et al 1980). However, little is known of the response of human peritoneal PAA to injury, particularly after bacterial inflammation. The use of fibrinolytic enhancement to reduce adhesions in man could only be considered when there has been confirmation that fibrinolytic activity is present in human peritoneum together with a greater understanding of the changes that occur in inflammation.

The aim of this study was to measure the fibrinolytic activity of normal human parietal peritoneum and omental visceral peritoneum, using tissue extraction of plasminogen activator, and to assess the effect of inflammation on this activity.

3.2 Materials and method

3.2.1 Peritoneal biopsies

Using the 6 mm punch biopsy, samples were obtained from normal parietal peritoneum and visceral peritoneum of the omentum of six patients undergoing elective surgery for non-inflammatory conditions. Biopsies were also obtained from the same sites in six patients undergoing surgery for acute appendicitis, as close as possible to the site of inflammation. The samples were obtained immediately after the peritoneal cavity had been opened, then wrapped in aluminium foil and frozen in dry ice before transfer to a freezer at -80°C. Patients gave informed consent to inclusion in this study, which was approved by the hospital ethical committee.

3.2.2 Determination of PAA

Plasminogen activator was extracted and peritoneal homogenates prepared and stored using the method previously described (Section 2.2.4). Samples were assayed on fibrin plates and quantified with rtPA standards incubated at the same time as described in Section 2.2.6. The lower limit of detection of PAA in this study was 0.06 IU/cm^2.

3.2.3 Statistical methods

The Mann-Whitney U test was used for comparisons between groups.

3.3 Results

The PAA of normal and inflamed parietal peritoneum is shown in Figure 3.1. The PAA of normal peritoneum was significantly higher compared with inflamed peritoneum (median 7.18 IU/cm^2 versus 0.08 IU/cm^2, $p<0.001$).

Similarly the PAA of normal omental visceral peritoneum was significantly higher than inflamed omental visceral peritoneum (median 4.59 IU/cm^2 versus 0.08 IU/cm^2, $p<0.01$) (Figure 3.2). There was no statistically significant difference in the PAA of normal parietal peritoneum and normal omental visceral peritoneum.

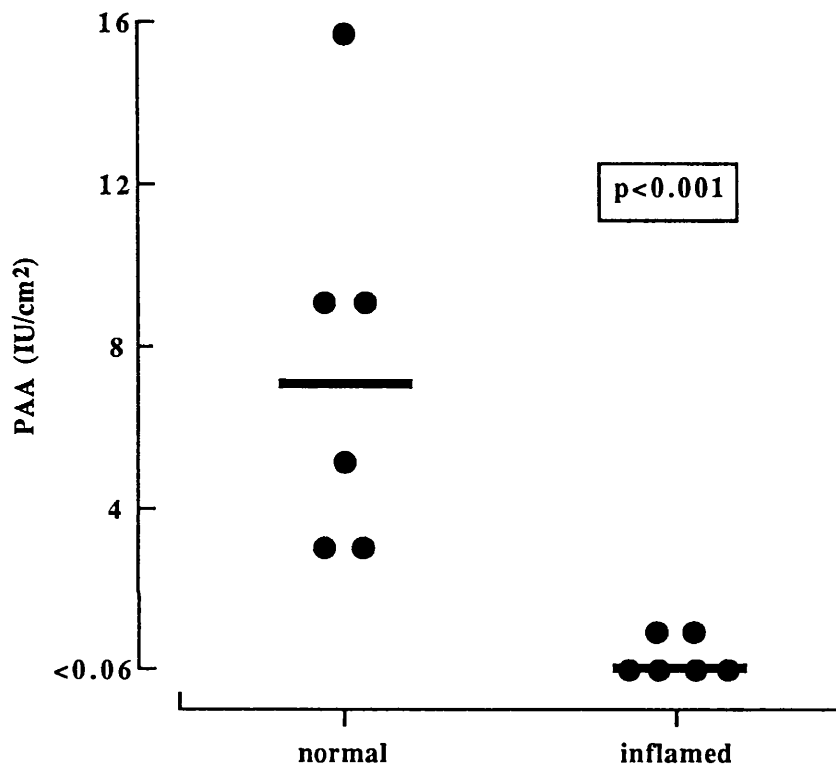

Figure 3.1 *Plasminogen activating activity (PAA) of normal and inflamed human parietal peritoneum (solid bar indicates median value)*

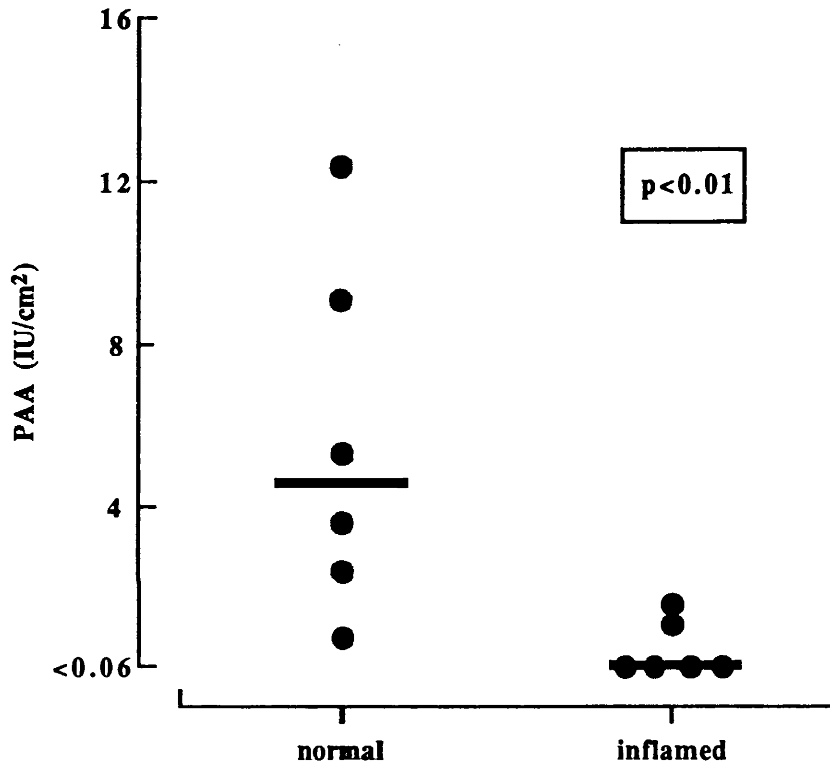

Figure 3.2 *Plasminogen activating activity (PAA) of normal and inflamed human omental visceral peritoneum (solid bar indicates median value)*

3.4 Discussion

Fibrinolytic activity of human peritoneum was first demonstrated by Porter et al in 1969 who incubated intact peritoneal samples on fibrin plates. *In vitro* abrasion of human peritoneum resulted in reduced zones of lysis of up to 40% compared with controls (Porter, Ball & Silver 1971; Gervin, Puckett & Silver 1973). Using the gelatin disc method, fibrinolytic activity was demonstrated in the parietal and visceral mesothelium of a wide variety of intra-abdominal organs (Merlo et al 1980). Although quantification of the assay was not performed, the greater omentum produced the largest zone of lysis. The presence of PAA in visceral peritoneum was confirmed by Corasanti et al (1980).

In the above studies only *in vitro* mechanical abrasion has been used to simulate injury of human peritoneum. Ischaemia and inflammation are considered the commonest predisposing causes of adhesions (see Chapter 1). The present study shows that inflammation results in significantly reduced peritoneal PAA *in vivo*. In a larger study performed in our department PAA was shown to be present in parietal and visceral peritoneum with little site to site variation (Thompson et al 1989). Additionally the PAA of ischaemic visceral peritoneum was significantly lower than normal visceral peritoneum. Direct comparison of these results with the present study is not possible as

the method employed by Thompson et al used urokinase standards for quantification.

The observation of reduced PAA in inflamed human parietal peritoneum and omental visceral peritoneum supports a pathophysiological explanation for adhesion formation: the reduced fibrinolytic activity of peritoneum limits lysis of the fibrinous adhesions associated with inflammation thus allowing organisation and the development of permanent adhesions. The reduction in peritoneal PAA observed in this human study may be related to mesothelial injury *per se*, diminished production of plasminogen activator, the presence of fibrinolytic inhibitors or any combination of these mechanisms. The time course of such a reduction in peritoneal PAA may also be important in determining the degree of organisation of fibrinous adhesions. Both these factors require further investigation.

CHAPTER 4

EFFECT OF EXPERIMENTAL PERITONITIS AND ISCHAEMIA ON PERITONEAL FIBRINOLYTIC ACTIVITY

4.1 Introduction

The fibrinolytic activity present in normal peritoneum may have an important physiological role in clearing fibrin deposited after an inflammatory stimulus. Reduction in PAA, after peritoneal injury in animals, has been reported and suggested that this is a common pathway to adhesion formation (Buckman et al 1976a). However in the majority of studies only mechanical injury (abrasion or peritoneal defects) has been examined and observations have been limited to PAA in the first 24 hours after injury. Adhesions are known to follow a wide variety of peritoneal insults and support for a common fibrinolytic pathway requires systematic chronological study of the effect of different forms of peritoneal injury on mesothelial fibrinolysis.

The aim of this experimental study was to determine the alteration in peritoneal PAA following standardised bacterial, chemical and ischaemic peritoneal injuries over the period of time during which adhesions are known to form.

4.2 Materials and method

4.2.1 Study design

One hundred and forty adult male Wistar rats (250-300g) were allocated equally to one of four groups: sham laparotomy, bacterial peritonitis, chemical peritonitis and peritoneal ischaemia. The operative procedure in all animals was performed under ether/air general anaesthesia using starch-free gloves (Biogel, Regent Laboratories, UK) and aseptic conditions. Following recovery of the animals at the end of the procedure they were allowed food (standard rat chow: CRM, Labsure Ltd., UK) and water *ad libitum*. A second laparotomy, again under general anaesthesia, was performed in five animals from each group at seven time intervals: 6 hours, 12 hours, 1, 4, 7, 10 and 14 days. At this second operation four 6 mm diameter biopsies were obtained from the parietal peritoneum of each animal. The biopsies were wrapped in aluminium foil, frozen in dry ice and transferred to a freezer at -80°C. An additional biopsy was taken from the bacterial and chemical peritonitis groups and the peritoneal ischaemia group and placed fresh in 10% formal-saline. Animals were killed, while still under anaesthesia, at completion of this second laparotomy by overdose of intravenous sodium pentobarbitone.

4.2.2 Sham laparotomy

Thirty five animals underwent a sham procedure whereby a 4cm midline abdominal incision was made. No intra-abdominal procedure or manipulation was performed and after ten minutes the wound was closed in two layers; continuous 3/0 PDS (Ethicon Ltd, Edinburgh, UK) to the muscle layer and continuous 3/0 Nurolon (Ethicon Ltd, Edinburgh, UK) to the skin.

Four peritoneal biopsies were also obtained from each of ten animals at time zero who had undergone no previous procedure and thus acted as baseline time zero controls.

4.2.3 Bacterial peritonitis

A bacterial inoculum was prepared by a modification of the method of Stewart & Matheson (1978). Five per cent w/v suspensions of homogenised fresh rat faeces in nutrient broth containing 10 per cent v/v glycerol (Sigma Chemical Co., Poole, UK) were prepared, filtered through surgical gauze and stored in 5 ml aliquots at -20°C. Previous work has shown that the bacterial count and viability is unchanged during storage at this temperature for three weeks (Stewart & Matheson 1978).

Under general anaesthesia 5 mls of the bacterial suspension, warmed in a water bath to 37°C, was injected intraperitoneally via a 20 gauge needle. At the time of re-laparotomy and peritoneal biopsy any intra-abdominal

adhesion or abscess was noted and a peritoneal swab taken. The initial inoculum and peritoneal swabs were analysed quantitatively for bacterial flora. Briefly, the samples were serially diluted in prereduced Hank's balanced salt solution (HBSS; Gibco, USA) and surface-plated in duplicate on blood agar, McConkey medium and Neomycin blood agar. Plates were prepared within the laboratory (agar from Oxoid Laboratories, UK; blood from Applied Protein Products, UK). Blood agar and McConkey plates were incubated aerobically at 37°C for 24 hours and all three plates were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) at 35°C for 48 hours. The number of viable bacteria were quantified in colony forming units per ml (CFU/ml). A count of $\geq 10^5$ CFU/ml was taken as significant growth.

4.2.3 Chemical peritonitis

A stock solution of 10 mg/ml tetracycline hydrochloride (Lederle Laboratories, Gosport, UK) was prepared. Under general anaesthesia, 5 ml of this tetracycline solution (warmed to 37°C) was injected intraperitoneally. At the time of re-laparotomy and peritoneal biopsy the presence of any adhesions was noted.

4.2.4 Peritoneal ischaemia

A midline laparotomy was performed and a 3 mm diameter ischaemic area of peritoneum created by ligation of a

button of parietal peritoneum with 3/0 silk (Ethicon Ltd., Edinburgh, UK). Six buttons were formed; three on each lateral peritoneal wall. At the time of re-laparotomy peritoneal biopsies were obtained from each animal immediately adjacent to an ischaemic button. Adhesions were scored as being present or absent to a peritoneal button, giving a quantified adhesion score of 0-6 for each animal.

4.2.5 Assay of PAA

Homogenates were prepared from each peritoneal biopsy and assayed for PAA using the fibrin plate method previously described (Section 2.2). Standard plates were prepared with standard solutions of rtPA. One sample from each time interval for each study group was incubated on fibrin plates that had been previously heated to 80°C for 20 min.

Due to the large number of samples assayed, several batches of fibrin plates were prepared and the lower limit of sensitivity for the assays ranged from 0.011 to 0.027 IU/cm^2.

4.2.6 Histological examination

The peritoneal biopsies preserved in formal-saline were fixed, cut and mounted. The sections were stained with Haemotoxylin and Eosin (H & E) and examined by a

pathologist who was unaware of the study group to which the biopsy belonged. The biopsies from the three experimental peritonitis groups were scored subjectively for the degree of acute or chronic inflammation. A sore of 0-3, (0 absent, 1 mild, 2 moderate and 3 severe), was allocated to the presence or absence of the following features: polymorphs, oedema, congestion and fibrin (acute inflammation); lymphocytes, monocytes, fibrosis and granulation tisssue (chronic inflammation).

4.2.7 Statistical methods

Kruskal-Wallis (non-parametric) two-way analysis of variance (ANOVA) was used throughout for comparison between groups.

4.3 Results

No lysis was observed on fibrin plates that had been heated to 80°C indicating that the lysis observed in all other plates was due to activation of plasminogen.

4.3.1 Sham laparotomy

The median peritoneal PAA of the ten control animals sampled at time zero was 0.11 IU/cm^2 (range 0.042 to 0.22 IU/cm^2).

At six and twelve hours post sham laparotomy there was no significant difference in peritoneal PAA compared with the PAA levels of the ten animals from time zero (Figure 4.1). However at one and four days there was a significant increase in PAA (median 0.26 IU/cm^2 for sham day 1 and 0.27 IU/cm^2 for sham day 4 versus 0.11 IU/cm^2 for time zero controls; $p<0.01$, ANOVA). The PAA of the sham animals remained higher than time zero control levels at two weeks.

The PAA levels at six and twelve hours after sham laparotomy were significantly different from the peak values found at one and four days and similarly these peak values were significantly greater than the level at fourteen days ($p<0.001$, ANOVA).

Adhesions were found in 4/35 (11%) animals and in all cases these were present after four days and had formed to the inner surface of the laparotomy wound.

4.3.2 Bacterial peritonitis

Results for the seven time intervals are shown in Figure 4.2. There was an initial decrease in PAA followed by a peak at four days and return to time zero control levels at two weeks. Compared with sham laparotomy the PAA at six and twelve hours was significantly lower (median 0.029 IU/cm^2 at 6 hr and 0.024 IU/cm^2 at 12 hr for bacterial peritonitis versus 0.112 IU/cm^2 at 6 hr and 0.112 at 12 hr for sham laparotomy; $p<0.0001$). The peak value of

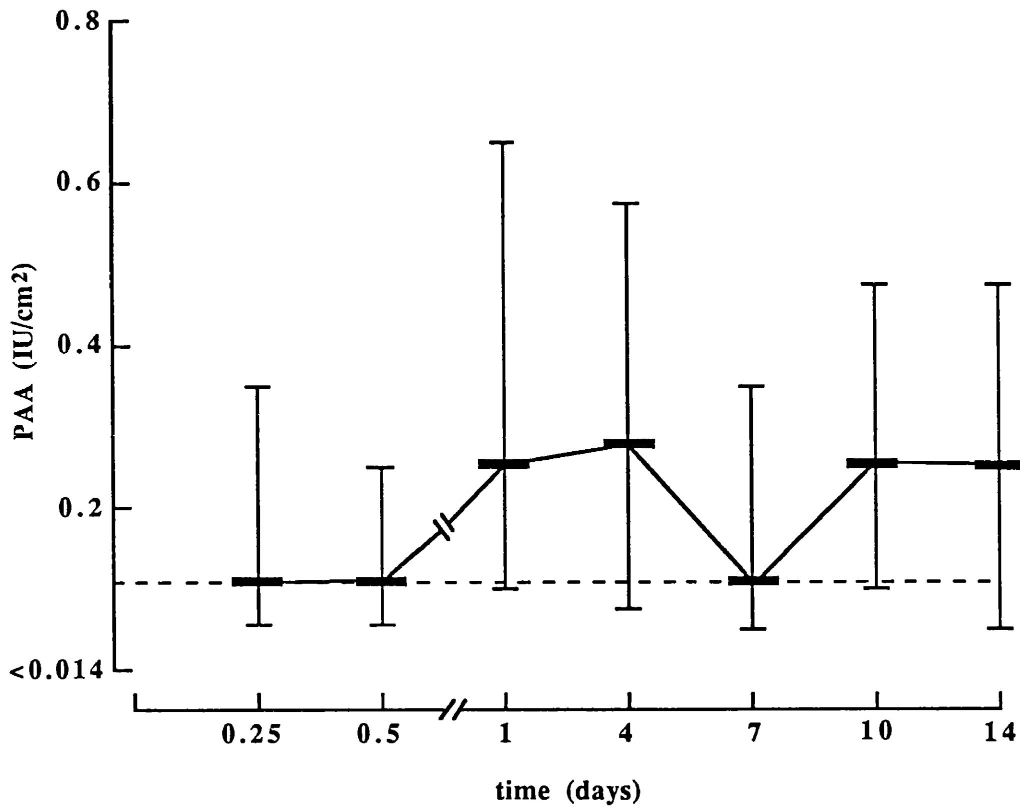

Figure 4.1 *Alteration of peritoneal plasminogen activating activity (PAA) with time following sham laparotomy (results shown as median and range; interrupted line indicates median PAA of rat peritoneal tissue at time zero)*

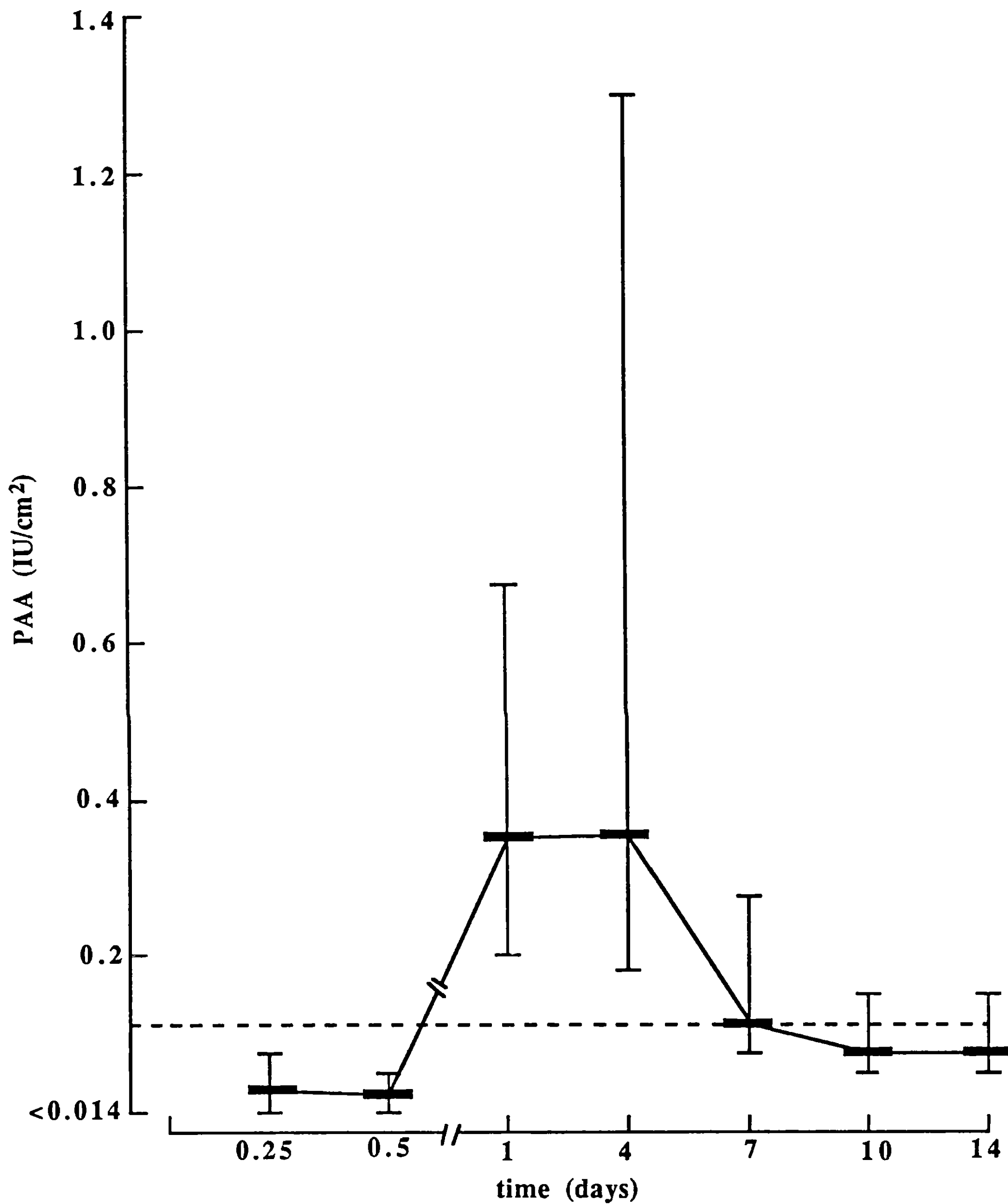

Figure 4.2 *Alteration of peritoneal plasminogen activating activity (PAA) with time following bacterial peritonitis (results shown as median and range; interrupted line indicates median PAA of rat peritoneal tissue at time zero)*

PAA at four days was significantly higher than the corresponding value for sham laparotomy (median 0.36 IU/cm^2 versus 0.27 IU/cm^2; $p<0.01$).

Intraperitoneal free fluid was present for up to 24 hours and adhesions noted in 13/35 (37%) animals (Table 4.1). Some of these adhesions incorporated a small abscess.

Microbiological assessment of the initial inoculum revealed a total aerobic count of 1.5 x 10^6 CFU/ml and anaerobic count of 4.6 x 10^6 CFU/ml. Peritoneal swabs were positive ($\geq 10^5$ CFU/ml) for coliforms and feacal-type streptococci up to four days and thereafter negative. Histological assessment (Table 4.2) showed a mild peritonitis compared with control peritoneum (Figures 4.3 & 4.4). This was reflected by no mortality in this group.

4.3.3 Chemical peritonitis

The profile of PAA against time is shown in Figure 4.5. Compared with sham laparotomy there was significant depression of PAA at 6, 12 and 24 hours (median 0.021 IU/cm^2 versus 0.112 IU/cm^2 at 6 hr, $\leq$0.014 IU/cm^2 versus 0.112 IU/cm^2 at 12 hr, 0.061 IU/cm^2 versus 0.263 IU/cm^2 at 24 hr; $p<0.0001$). The peak at 4 days was significantly higher than sham laparotomy (median 0.69 IU/cm^2 versus 0.27 IU/cm^2; $p<0.0001$) and at 14 days the PAA level was still higher (median 0.47 IU/cm^2 versus 0.184 IU/cm^2; $p<0.001$).

Table 4.1 *Adhesion formation in each study group (number of animals forming adhesions in each study group at each time interval: maximum = 5)*

	6 hrs	12 hrs	1 day	4 day	7 day	10 day	14 day
sham laparotomy	0	0	0	1	0	1	0
bacterial peritonitis	0	0	0	3	3	4	3
chemical peritonitis	0	2	3	3	2	4	4
peritoneal ischaemia	5	5	5	5	5	5	5

Table 4.2 *Acute and chronic inflammation score for the bacterial, chemical and ischaemia groups at each time interval.*
(Each value represents total inflammation score: maximum score = 12 for acute inflammation; 9 for chronic inflammation)

	6 hrs	12 hrs	1 day	4 day	7 day	10 day	14 day
Acute inflammation							
bacterial peritonitis	4	2	3	2	0	0	0
chemical peritonitis	2	7	8	2	3	1	1
peritoneal ischaemia	7	6	8	4	2	2	1
Chronic inflammation							
bacterial peritonitis	0	2	1	2	1	1	0
chemical peritonitis	0	1	2	1	4	5	6
peritoneal ischaemia	0	0	1	5	3	9	9

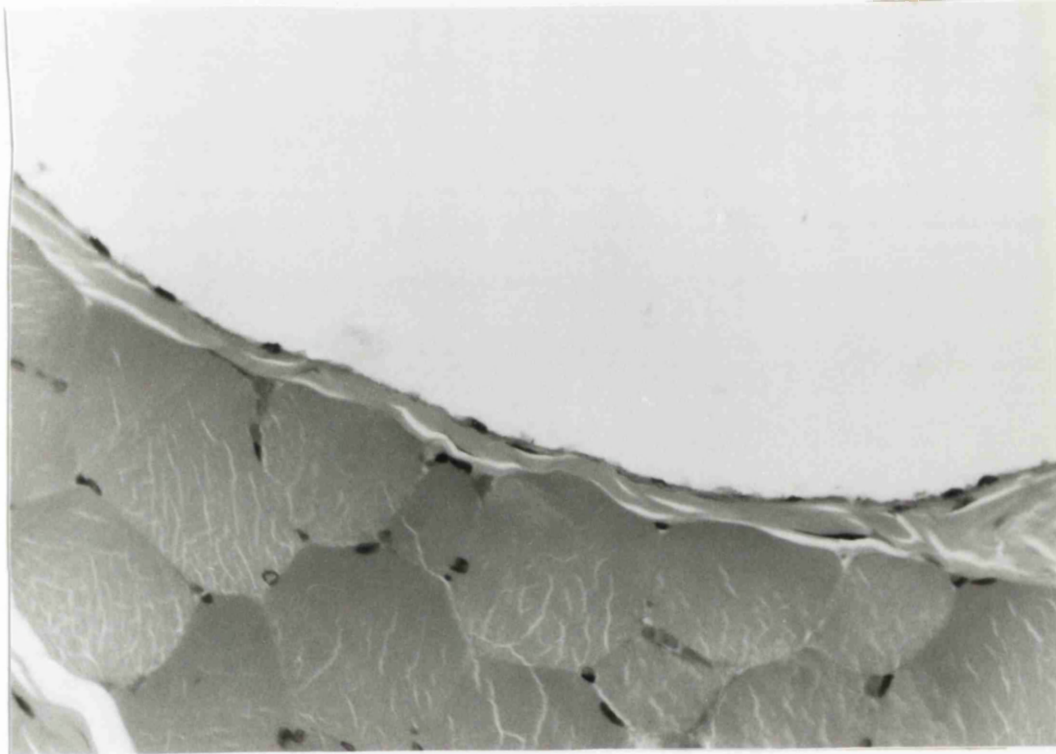

Figure 4.3 *Normal rat peritoneum from time zero control (H & E, x40). The peritoneal surface is formed by flattened mesothelial cells.*

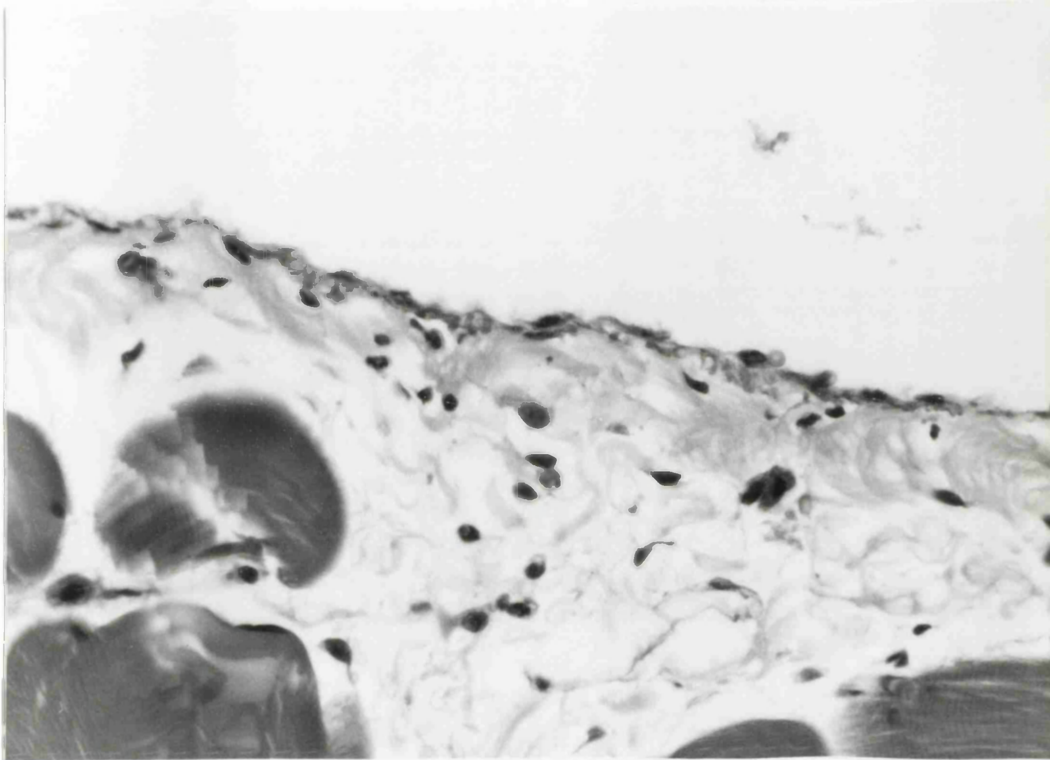

Figure 4.4 *Rat peritoneum following bacterial peritonitis (12 hours, H & E, x40). The mesothelial cells are more rounded with oedema of the underlying connective tissue.*

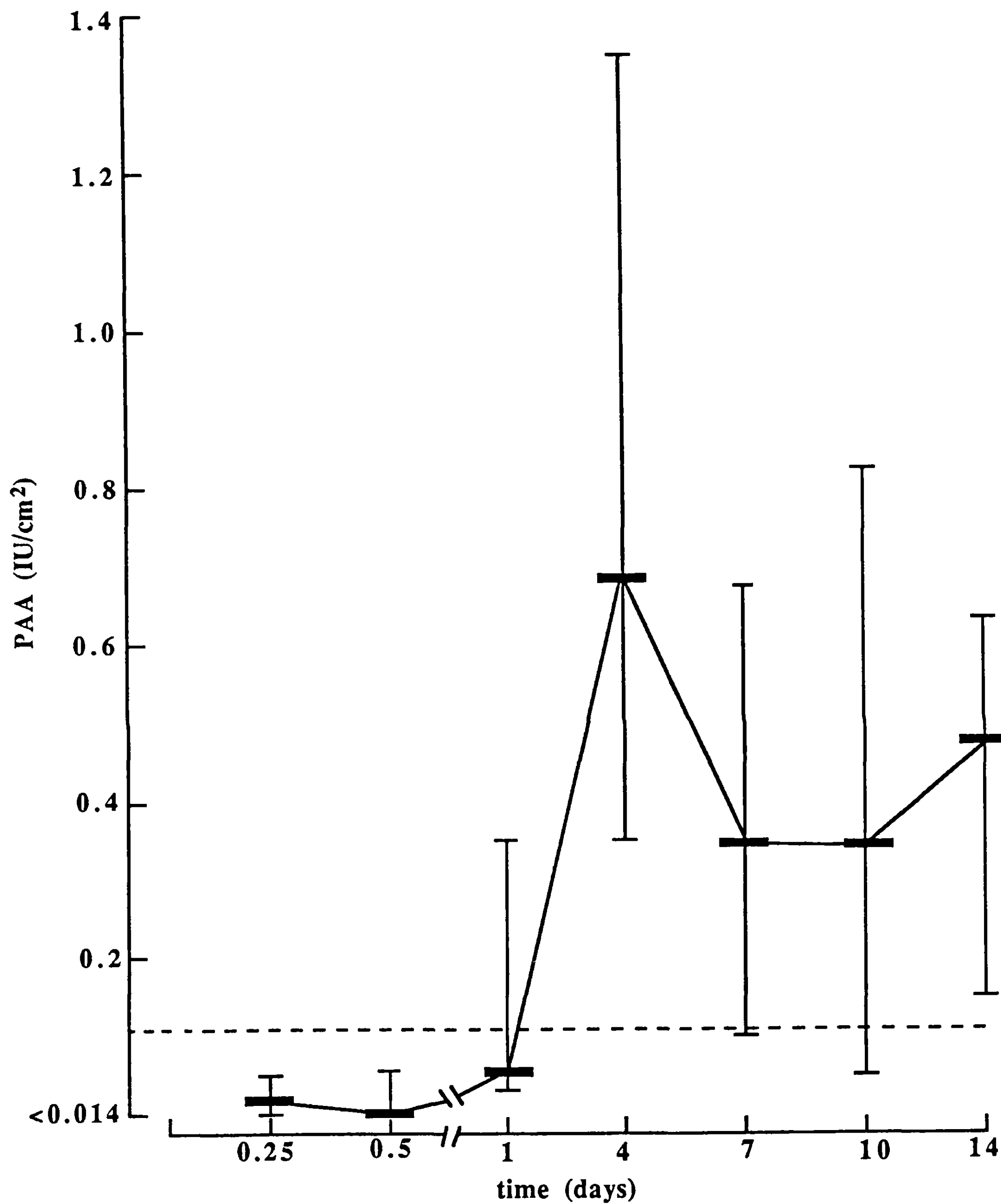

Figure 4.5 *Alteration of peritoneal plasminogen activating activity (PAA) with time following chemical peritonitis (results shown as median and range; interrupted line indicates median PAA of rat peritoneal tissue at time zero)*

Adhesions formed in 22/35 (63%) animals (Table 4.1). They were noted from 12 hours, although adhesions at this time were flimsy and easily separated. On histological grounds the peritoneal inflammation was more severe than in the bacterial peritonitis group (Table 4.2, Figure 4.6).

4.3.4 Peritoneal ischaemia

Median PAA values for the seven time intervals are shown in Figure 4.7. Compared with the sham group the reduction in PAA at six and twelve hours was statistically significant (median 0.05 IU/cm^2 versus 0.112 IU/cm^2 at 6 hr, 0.05 IU/cm^2 versus 0.112 IU/cm^2 at 12 hr; $p<0.001$). The rise in PAA seen at 4, 7 and 10 days was also significantly higher than the sham group (median 0.71 IU/cm^2 versus 0.27 IU/cm^2 at 4 days, 0.89 IU/cm^2 versus 0.112 IU/cm^2 at seven days, 0.43 IU/cm^2 versus 0.256 IU/cm^2 at ten days; $p<0.01$).

Adhesions formed in all animals (Table 4.1). The median adhesion score (number of ischaemic buttons per animal with an adhesion) was three at six hours; five at 24 hours and remained at five up till two weeks (Figure 4.8). Up to 24 hours adhesions were flimsy and easily separated but gradually became firmer so that at two weeks they could only be separated by sharp dissection (Figure 4.9).

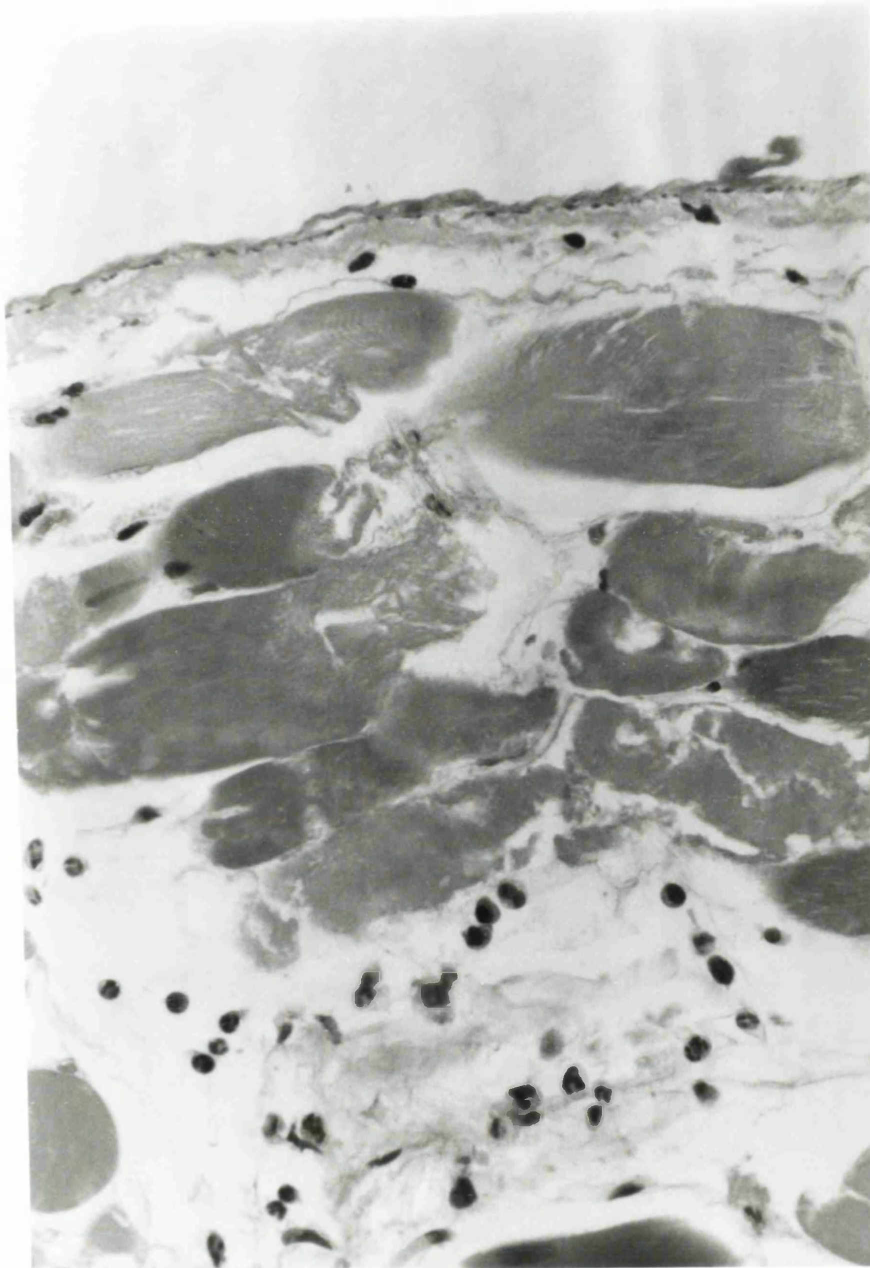

Figure 4.6 *Rat peritoneum following chemical peritonitis (12 hours, H & E, x40). There is loss of the mesothelial surface with oedema of the underlying connective tissue and the presence of acute inflammatory cells.*

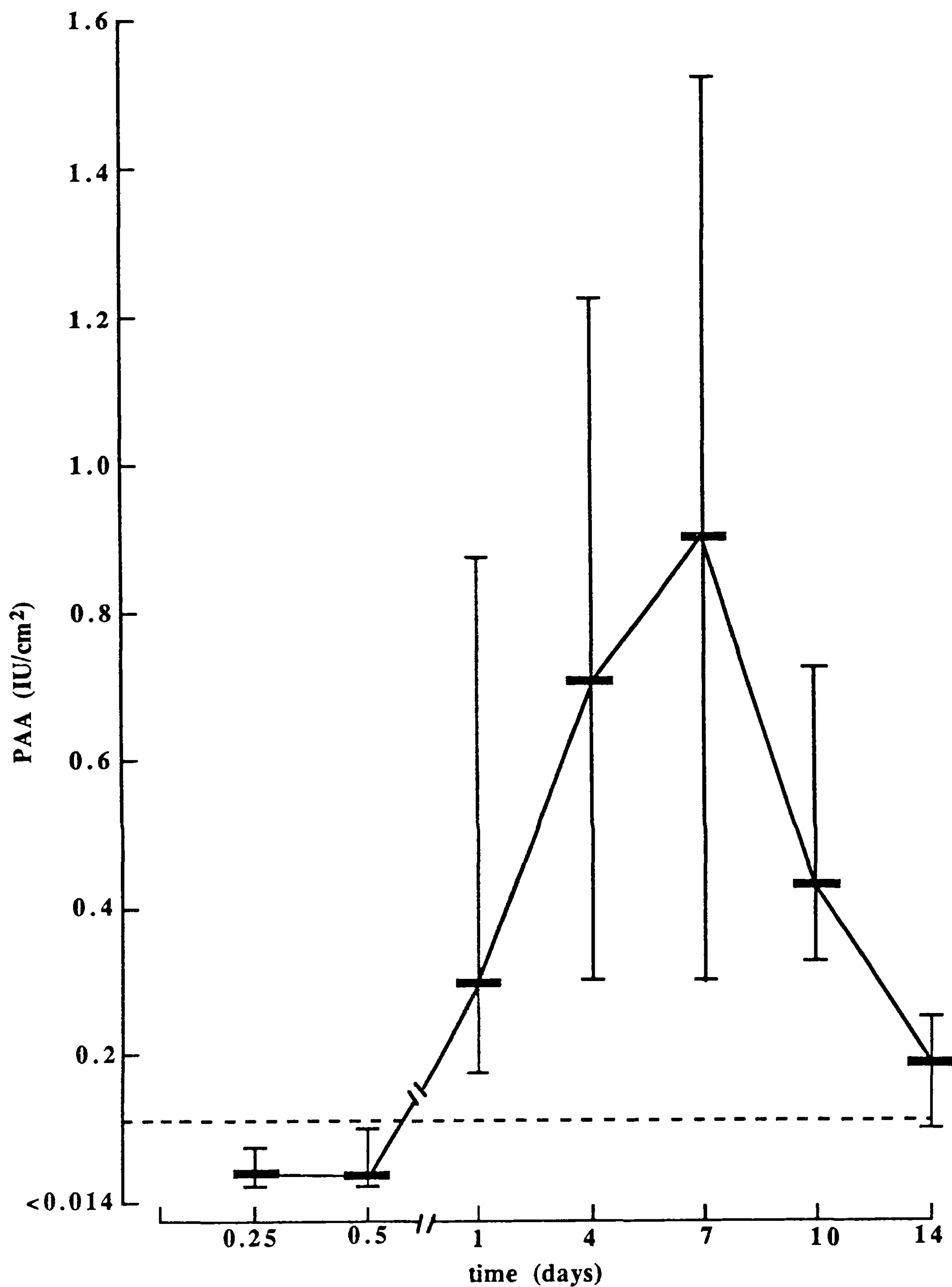

Figure 4.7 *Alteration of peritoneal plasminogen activating activity (PAA) with time following peritoneal ischaemia (results shown as median and range; interrupted line indicates median PAA of rat peritoneal tissue at time zero)*

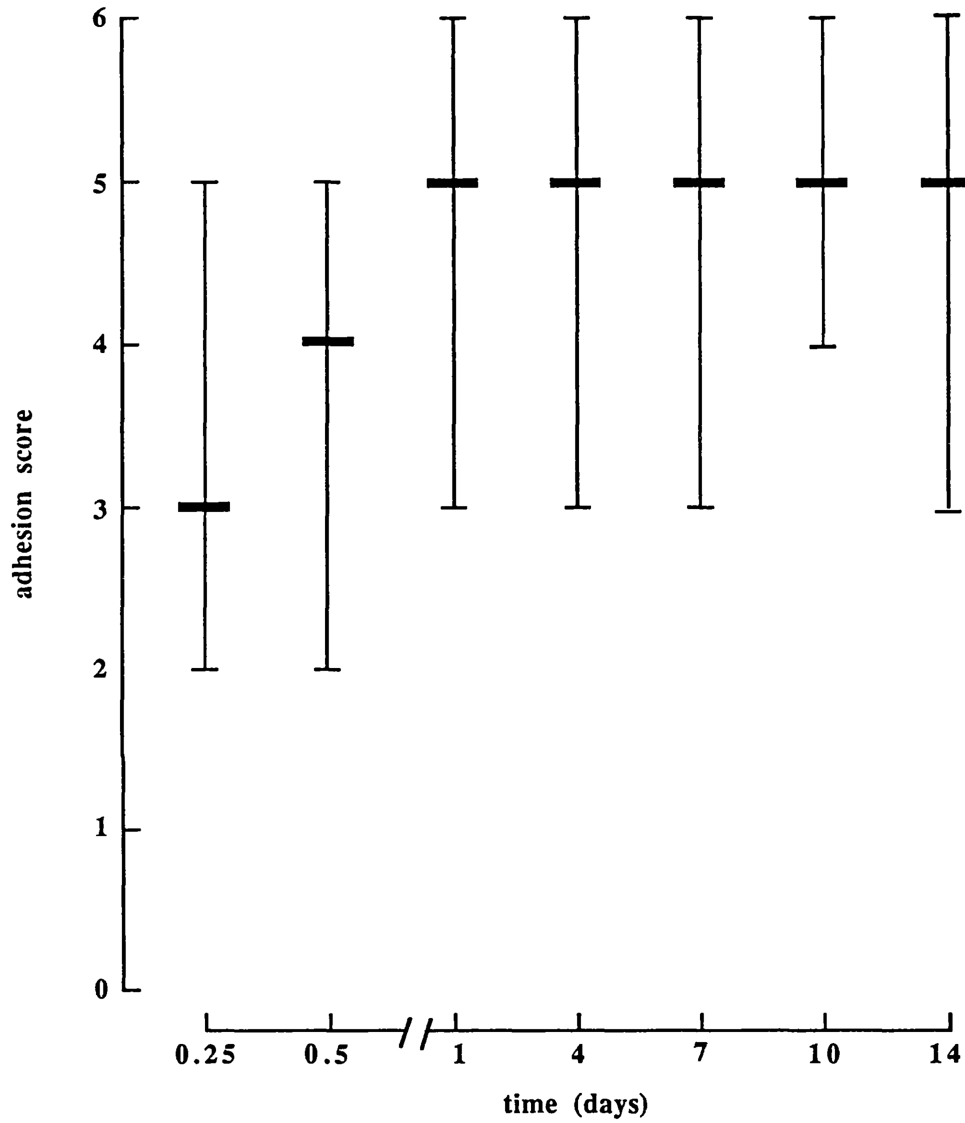

Figure 4.8 *Quantitative adhesion score for peritoneal ischaemia for the seven time intervals (median and range)*

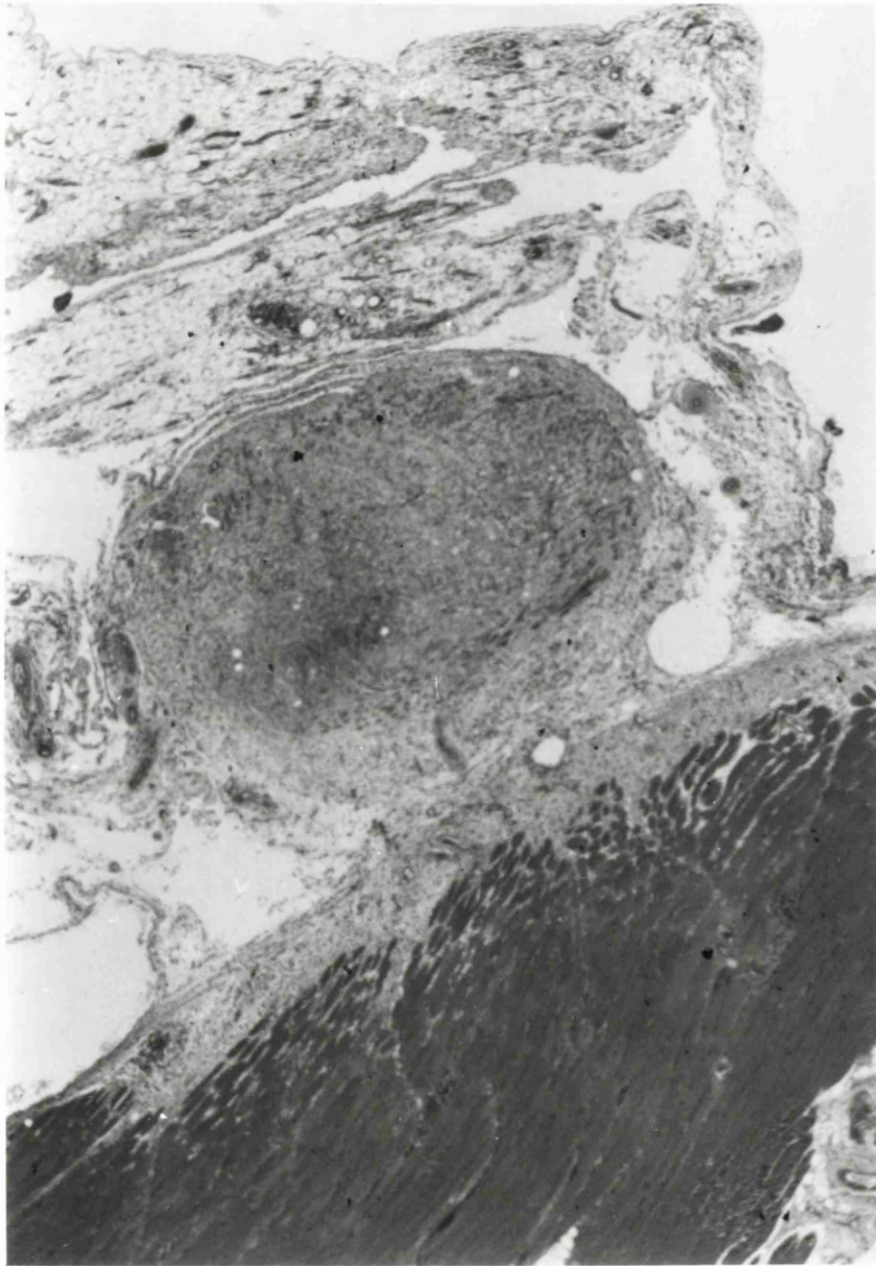

Figure 4.9 *Mature adhesion to ischaemic peritoneal button at two weeks (H & E, x2.5).*

4.4 Discussion

In the first 24 hours after all three types of injury there was a three-fold or greater depression of PAA compared with the sham procedure. This is similar to the findings after mechanical abrasion of dog parietal peritoneum (Porter et al 1969) or ileum (Gervin, Puckett & Silver 1973), where whole tissue assayed almost immediately after the procedure showed a greater than 50% decrease in PAA. Hau, Payne & Simmons (1979) in the dog and Raftery (1981b) in the rat created an ischaemic segment of ileum to induce bacterial peritonitis. At 24 hours PAA of the parietal peritoneum was abolished in both studies. There was a high mortality from this model of peritonitis and no further measurements of PAA were made. In our study introduction of a non-lethal inoculum depressed, but did not abolish, PAA and this may be due to the milder degree of peritonitis induced as evidenced by histological assessment and no mortality. Also, in the studies of Hau et al and Raftery, a significant operative procedure was performed and this mechanical trauma may have exerted an additional depression of PAA.

The effect of chemical peritonitis was to produce a large reduction in PAA with no functional activity present at 12 hours. Comparison of the histology of the bacterial and chemical injuries revealed the greater severity of the latter. A previous study using 10 mg/ml tetracycline

intraperitoneally also found marked peritoneal damage with complete loss of mesothelial microvilli and only partial recovery at 72 hours associated with adhesion formation in all animals (Phillips & Dudley 1984). Phenol or formaldehyde instilled into the peritoneal cavity of dogs have also been found to abolish PAA when this was measured immediately after injury (Porter et al 1969).

Ischaemic grafts of peritoneum have been produced in the dog (Buckman et al 1976b) and rat (Raftery 1981b) resulting in reduced fibrinolytic activity at 24 hours in biopsies taken from the graft. In the study by Raftery areas of diathermy, which produces a combination of thermal and ischaemic injury, showed no PAA at 24 hours.

This reduction in PAA which is seen universally after peritoneal injury may be due to decreased levels of plasminogen activator or inactivation of this enzyme. There is some evidence that the latter occurs. Incubation on a fibrin plate of biopsies of inflamed peritoneum around a biopsy of normal peritoneum, known to have fibrinolytic activity, resulted in inhibition of the zone of lysis around the normal sample (Buckman et al 1976b). Similarly mesothelial suspensions from traumatised bovine peritoneum prevented spontaneous lysis of fibrin plates. This effect was blocked by prior heating of the mesothelial suspension which suggested the presence of an inhibitor of plasminogen activator within the mesothelium (Pugatch & Poole 1969).

None of the above studies examined peritoneal PAA as recovery from injury took place. The rebound peak of PAA found in the present study occurred with all three insults. It is also of note that the same profile was seen in the sham group though without the initial reduction in PAA. A similar observation was made by Ryan, Grobéty & Majno (1973) who looked at peritoneal PAA after caecal drying. The fibrin slide method was used, so accurate quantification of fibrinolytic activity was not possible, but no lysis was observed at 24 hours followed by larger zones than normal at 5 days and then a return to normal levels.

The presence of this rebound phenomenon in all four groups suggests that it is an integral part of the mesothelial response to injury. The timing of its occurrence, between one and seven days, coincides with the period when organisation of fibrinous adhesions occurs and thus may be a physiological response seen to enhance lysis of fibrin at this time. The mechanism underlying this effect can only be speculated upon. During the early healing after peritoneal injury new capillaries are formed in granulation tissue. It is known that these are a rich source of plasminogen activator and they may provide an excess of this enzyme. However, in the sham laparotomy group, no direct peritoneal injury occurred to set in train the events which would lead to the formation of granulation

tissue in, for example, the region of peritoneal biopsy. Also in the bacterial and chemical peritonitis groups, where histological assessment was performed, granulation tissue was not a dominant feature.

In addition to the sham group where there is no direct peritoneal injury, the rebound phenomenon is seen in the ischaemia group where biopsies were taken from peritoneum adjacent to the injury and not from the button itself. The liagation of a peritoneal button may have produced some ischaemia of surrounding tissue and thereby induced a "secondary" inflammatory reaction. Ischaemic tissue is also known to produce abundant oxgen-derived free radicals (Parks et al 1982). These may contribute to increased permeability of ischaemic tissue, leading to exudation of fibrinogen and the formation of fibrinous adhesions (O'Leary et al 1988). This hypothesis has received support from the observation that free radical "scavengers" given intravenously to rats after ileal vascular obstruction reduced the severity of adhesions (Tsimoyiannis et al 1989). It is also possible that there may be autocrine regulation of peritoneal PAA whereby mesothelial cells adjacent to an area of injury respond to factors released by the injured cells. It has been shown in studies on vascular endothelium that inhibitors of plasminogen activator provide a feedback mechanism leading to increased production of plasminogen activator (Collen 1988). Thus

the possibility exists that the initial reduction of PAA seen after injury is due to inhibition of plasminogen activator and that local factors or the inhibitors themselves then result in increased plasminogen activator production and subsequent return of peritoneal PAA as part of a feedback loop as mesothelial recovery occurs.

Adhesions occurred to a variable degree after all three types of injury. More adhesions were seen in the chemical peritonitis group, where the depression of PAA was more profound and lasted longer than the bacterial group. Adhesions formed in all animals with ischaemic buttons but it is not possible to relate their formation directly with reduced PAA of the button itself. The presence of suture material may also provide a stimulus in this group although studies have shown that a ligature placed loosely through peritoneum without strangulating tissue does not lead to adhesion formation (Ellis 1982).

Possible explanations for the time-profile of PAA after injury can be only speculative. However the study performed shows that PAA was reduced significantly with a secondary increase as recovery occured. The same pattern was seen in three distinct forms of insult and was associated with adhesion formation. This supports the hypothesis that reduced fibrinolytic activity is a common pathway to adhesion formation. The degree of PAA reduction and timing of recovery is reflected by the severity of the

injury. This may explain why adhesions form to a variable degree depending on the severity and duration of the initiating insult - these factors altering the balance between fibrinolysis and the organisation of fibrin deposits. The present study also indicates that agents which may be used to enhance peritoneal fibrinolysis in an attempt to prevent adhesion formation would need to be effective for at least 24 hours.

CHAPTER 5

IDENTIFICATION OF THE PLASMINOGEN ACTIVATOR IN HUMAN PERITONEUM

5.1 Introduction

The determination of plasminogen activating activity by the fibrin plate method measures functional fibrinolytic activity. The absence of lysis on preheated plates indicates that the fibrinolytic activity of normal human peritoneum (described in Chapter 3) is mediated by activation of plasminogen but does not differentiate the type of plasminogen activator present. The two principal physiological plasminogen activators are tPA and uPA which have both been demonstrated in a wide variety of tissues (Chapter 1, Section 1.7). The aim of this study was to distinguish which of these two plasminogen activators mediates plasminogen activating activity in human peritoneum.

5.2 Material and methods

5.2.1 Peritoneal samples

Biopsies of normal non-inflamed parietal peritoneum from six patients undergoing elective surgery were obtained with a 6 mm diameter punch biopsy. Extraction of plasminogen activator and preparation of peritoneal homogenates were by the method described previously (Section 2.2.4). The six homogenates were stored at -80°C until assay.

5.2.2 Determination of plasminogen activator

Antibodies to human tPA and uPA may be raised in a number of animals. The specific antibody, by binding to tPA or uPA, will inactivate the enzyme. Normal human peritoneum is known to possess plasminogen activating activity (Chapter 3). Incubating anti-tPA or anti-uPA antibody with homogenates of normal human peritoneal tissue will inactivate any tPA or uPA present in the homogenate. The homogenates will then show no plasminogen activating activity on fibrin plates. If an antibody has strong specificity for the plsminogen activator in human peritoneum then inhibition of PAA will occur with a low concentration of the antibody. By using serial dilutions of each antibody with peritoneal homogenates of known PAA, a point at which 50% of normal PAA is inhibited may be found. This principle was used in determining the major plasminogen activator in human peritoneum.

Lyophilised polyclonal goat anti-tPA antibody and anti-uPA antibody (Biopool, Umeå, Sweden) were reconstituted with 1 ml of buffer (20 mM sodium dihydrophosphate, 100 mM sodium chloride; pH 7.3). The reconstituted antibody was serially diluted with 100 µl aliquots of buffer to produce a series of 16 dilutions of anti-tPA and anti-uPA antibody ranging from a titre of 1 in 2 to 1 in 65 536.

Tissue homogenates from the six samples of normal

peritoneum were brought to room temperature. Twenty µl from each of the samples was incubated with a 20 µl aliquot from each of the serial dilutions of anti-tPA antibody and anti-uPA antibody for 30 min at 37°C. Plasminogen-containing fibrin plates were prepared by a standard method with a 6 mm well cut in the centre of the plate (Section 2.2.5). An aliquot (20 µl) from each peritoneal homogenate/antibody dilution sample was placed in the central well of a fibrin plate.

The following controls were also performed: dilution buffer alone; anti-tPA antibody alone; anti-uPA antibody alone; peritoneal homogenate with normal goat serum (all 20 µl samples). To determine the PAA of the peritoneal samples, 20 µl of each peritoneal homogenate alone was placed on a fibrin plate in duplicate. Solutions of rtPA of known activity were incubated on fibrin plates to produce a standard curve.

All the fibrin plates were then incubated for 24 hours at 37°C and the diameter of the zone of lysis around the central well measured directly. From the standard curve, the PAA level of the control and sample solutions was calculated (Section 2.2.6)

For the samples incubated with anti-tPA antibody and anti-uPA antibody the titre giving ≥50% inhibition of fibrin plate lysis compared with the peritoneal homogenates alone was calculated.

5.2.3 Assay of tPA

The level of tPA antigen in the peritoneal homogenates alone was determined by enzyme-linked immunosorbent assay (Tintelize, Fluorochem, Old Glossop, UK). The technique is well established and has been detailed by Korninger et al (1986). Briefly, 20 µl samples of peritoneal homogenate were added to micro-test wells. These were pre-coated with 2 µg goat monoclonal anti-human tPA IgG which binds the tPA present in the sample. A second goat monoclonal anti-tPA antibody conjugated to the enzyme peroxidase was added. The antibody conjugate binds to other free antigenic determinants on the tPA, immobilized on the wells by the coat antibody, thus forming a "sandwich": coat-antibody to tPA to antigen-conjugate antibody. Unbound conjugate (and all other unbound material) was washed away and a peroxidase enzyme substrate (ortho-phenylenediamine) added. The substrate yields a yellow colour upon rection with the peroxidase-conjugated antibody. The amount of colour is directly proportional to the amount of tPA in the sample and was read on a spectrophotometer at 492 nm.

5.2.4 Statistical methods

For graphic presentation of antibody titres, $-\log_2$ was used so that titres could be expressed as linear integers without unwieldy large numbers. For example $-\log_2 1$ indicates a titre of 1 in 2; $-\log_2 2$ a titre of 1 in 4 and

so on to $-\log_2 16$ representing a titre of 1 in 65 536. The Mann-Whitney U and Spearman rank correlation tests were used.

5.3 Results

5.3.1 Control samples

The control samples: dilution buffer alone, anti-tPA antibody alone and anti-uPA antibody alone produced no lysis. The control samples of peritoneal homogenate with normal goat serum had a similar PAA to peritoneal homogenate alone (median 4.75 IU/cm^2 versus 4.59 IU/cm^2). This indicates that goat serum contained no additional plasminogen activating activity or significant inhibitors of fibrinolysis.

5.3.2 Determination of plasminogen activator

The antibody titre required to inhibit PAA by 50% or greater is shown in Figure 5.1. A median titre of 1 in 6 144 ($2^{12.5}$) of anti-tPA antibody was required to inhibit the PAA of normal peritoneum. This contrasts with a median titre of 1 in 4 (2^2) of anti-uPA antibody required to achieve the same degree of inhibition ($p<0.01$).

The median PAA of the peritoneal homogenates was 4.59 IU/cm^2 (range 3.0 to 12.05) and the median tPA antigen

level 1.28 ng/ml (range 0.87 to 2.36). Correlation of these two parameters is shown in Figure 5.2 (correlation coefficient 0.87, $p<0.05$).

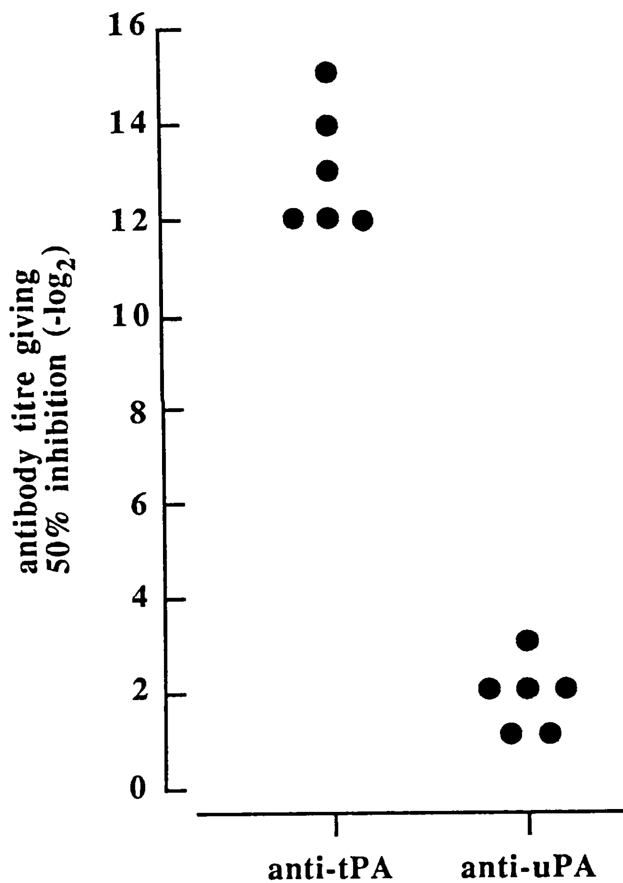

Figure 5.1 *$-log_2$ titre of anti-tissue plasminogen activator antibody (anti-tPA) and anti-urokinase plasminogen activator antibody (anti-uPA) giving ≥50% inhibition of plasminogen activating activity in normal human peritoneum*

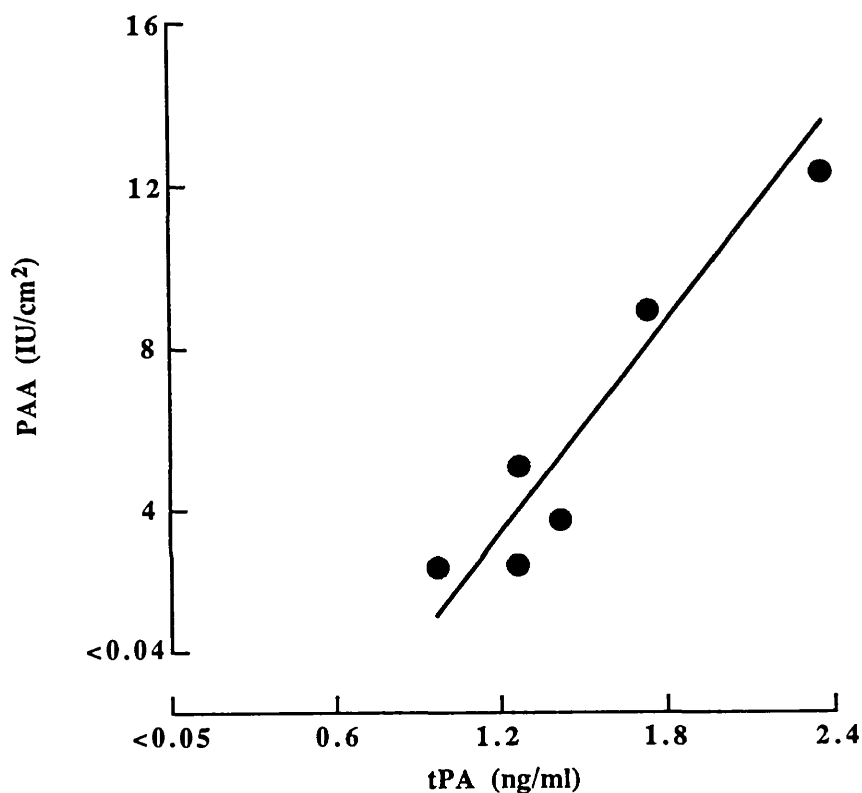

Figure 5.2 *Relationship between concentration of tissue plasminogen activator (tPA) with plasminogen activating activity (PAA) of normal human peritoneum (n=6, r=0.87, p<0.05).*

5.4 Discussion

Very low concentrations of anti-tPA antibody inhibited human peritoneal fibrinolytic activity. There was also some inhibition of activity with anti-uPA antibody but at a much higher concentration (1 in 4 dilution). This effect is unlikely to represent a significant presence of uPA in peritoneum as the antibodies used were of polyclonal origin and the anti-uPA antibody at such a high concentration might have inhibited tPA. The disparity in efficacy of anti-tPA and anti-uPA in inhibiting plasminogen activating activity, together with the correlation between tPA antigen concentration and overall functional peritoneal PAA, provides strong evidence that tPA is the physiological plasminogen activator in human peritoneum.

The slight discrepancy between tPA activity detected by ELISA and by fibrin plate assay is to be expected. The immunoassay measures both free and bound tPA whilst the functional bioassay measures only the active or free tPA. Within plasma it is known that tPA circulates in both an active and inactive form. The presence of a good correlation between the tPA concentration and PAA suggests that, in normal non-inflamed peritoneum, the majority of the tPA present is in an active form.

The precise site of tPA production in human peritoneum has not been studied. It has been established that the endothelial cell is the major source of plasma tPA

(Kristensen et al 1984). The mesothelial cell is morphologically very similar and thus is a likely source. It is though possible that sub-mesothelial capillaries may produce tPA which is then transported via the mesothelium to the peritoneal cavity. The gene sequence for human tPA has been identified and from this cDNA probes developed (Pennica et al 1983). By employing *in-situ* hybridisation techniques for messenger RNA such probes could be used to localise the cellular site of tPA production in human peritoneum.

The fibrin-specific action of tPA makes it an ideal enzyme for the role of peritoneal fibrinolysis. Following peritoneal injury the inflammatory exudate which is generated will contain fibrin and, as part of the plasma proteins, plasminogen. Peritoneal tPA is able to complex with this free fibrin and plasminogen in a ternary complex localising fibrinolysis to any fibrinous deposits. The presence of tPA in normal peritoneum suggests that it may also play a role in the uninjured state preventing adhesion formation to its mesothelial surface. The peritoneal surface is normally covered by a thin fluid layer and the presence of tPA within the fluid would add a potental fibrinolytic activator to this lubricant barrier.

CHAPTER 6

MOLECULAR MECHANISM OF REDUCED HUMAN PERITONEAL FIBRINOLYTIC ACTIVITY IN INFLAMMATION

6.1 Introduction

The animal studies of different forms of peritonitis described in Chapter 4 showed that the PAA of peritoneum, a measure of its potential functional fibrinolytic activity, was decreased in inflammation and this was associated with adhesion formation. Elucidation of the underlying molecular mechanism responsible for this diminished activity would be of value in developing therapies to enhance fibrinolysis at the appropriate time and so possibly prevent adhesions. The decrease in PAA may be due to diminished levels of tPA, which has been shown to be the specific plasminogen activator in human peritoneum, or to inhibition of the fibrinolytic system, or a combination of both mechanisms.

Specific inhibitors are known to function within the vascular system to regulate the fibrinolytic process (discussed in Section 1.7). Whilst understanding of the regulation of fibrinolysis by these inhibitors is incomplete, current knowledge indicates that PAI-1 is the principal physiological inhibitor of tPA and α_2-antiplasmin of plasmin (Sprenngers & Kluft 1987). The aim of this study was to determine the changes in fibrinolytic enzymes in peritoneal inflammation; specifically to relate the relative roles of the known activator in peritoneum, tPA, with the two principal inhibitors α_2-antiplasmin and PAI-1.

6.2 Materials and methods

6.2.1 Peritoneal samples

Peritoneal biopsies were taken with a 6 mm diameter biopsy punch from the parietal peritoneum of patients undergoing abdominal surgery. Normal peritoneum from the anterior abdominal wall was obtained from ten patients undergoing elective surgery (cholecystectomy, 6; gastrointestinal resection, 4) and inflamed peritoneum from ten patients (appendicitis, 7; perforated viscus, 3).

Peritoneal homogenates were prepared by the method descibed in Section 2.2.4. and stored at -80°C until assay.

6.2.2 Fibrinolytic system component assays

The tissue homogenates from all normal and inflamed peritoneal biopsies were brought to room temperature. For each of the samples the following assays were performed in duplicate. Plasminogen activating activity was determined by the fibrin plate technique; at the same time standard solutions of recombinant tPA were placed on fibrin plates. The plates were incubated at 37°C for 24 h and the zone of lysis measured as described before. From the standard curve of diameter of fibrin plate lysis against $\log_{10}$ rtPA the PAA of the normal and inflamed specimens was calculated. The lower limit of sensitivity of the assay

was 0.04 IU/cm^2.

Both tPA and PAI-1 were measured by enzyme-linked immunosorbent assay (Tintelize, Biopool, Fluorochem Ltd, UK). Alpha-2-antiplasmin was measured by chromogenic assay (KabiVitrum, Hillingdon, UK). In this latter assay a 20 µl aliquot of peritoneal homogenate was incubated with an excess of plasmin. Any α_2-antiplasmin present in the sample inactivated plasmin; the remaining plasmin was measured by action on a chromogenic substrate. Plasmin cleavage of this substrate releases p-nitroaniline which can be measured spectrophotometrically.

The lower limits of sensitivity for the assays were 0.05 ng/ml (tPA), 2.5 ng/ml (PAI-1) and 0.05 µM/l (α_2-antiplasmin).

6.2.3 Statistical method

The Mann-Whitney U test was used for comparisons between groups.

6.3 Results

The PAA of normal and inflamed peritoneum determined by fibrin plate lysis is shown in Figure 6.1. For inflamed peritoneum PAA was almost completely abolished compared with normal peritoneum (median ≤ 0.07 IU/cm^2 vs. 12.0 IU/cm^2; $p<0.001$).

There was no statistically significant difference in the levels of tPA antigen in normal and inflamed peritoneum (Figure 6.2). Median tPA level for normal peritoneum was 1.03 ng/ml compared with 0.97 ng/ml for inflamed peritoneum.

Alpha-2-antiplasmin was not detected in either normal or inflamed peritoneum. The results for PAI-1 are shown in Figure 6.3. In normal peritoneum PAI-1 antigen was not detected but in inflamed peritoneum it was universally present (median ≤ 2.5 ng/ml versus 8.45 ng/ml; $p<0.001$).

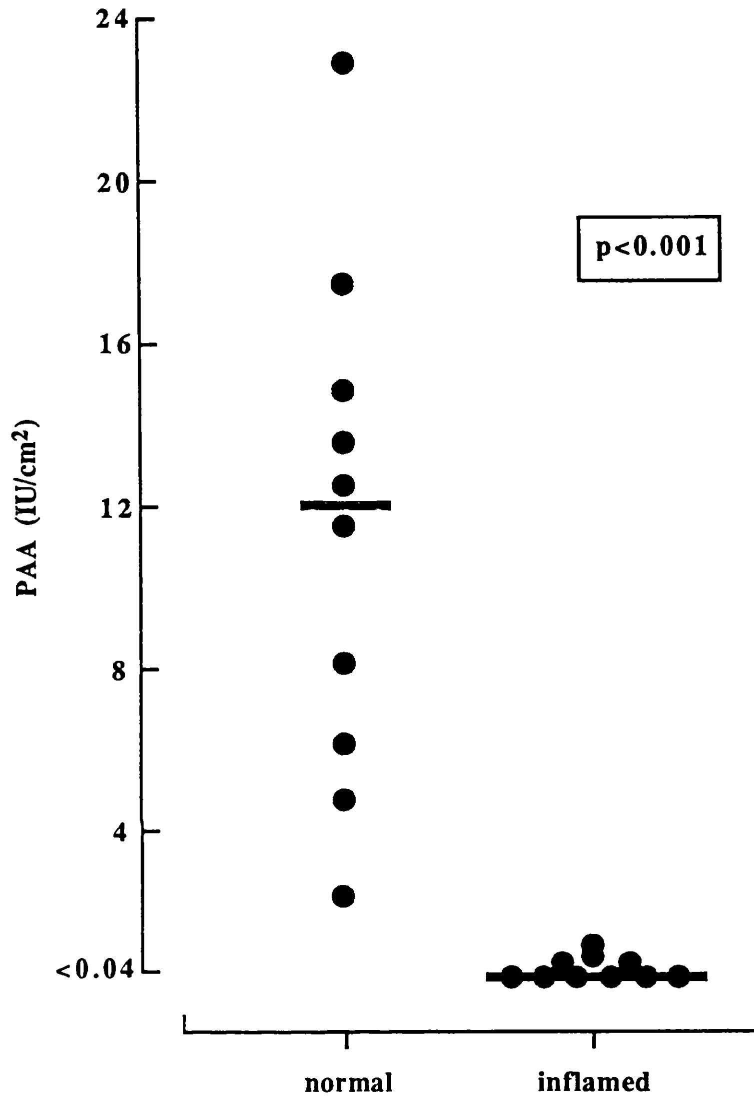

Figure 6.1 *Plasminogen activating activity (PAA) of normal and inflamed human peritoneum (solid bar indicates median value)*

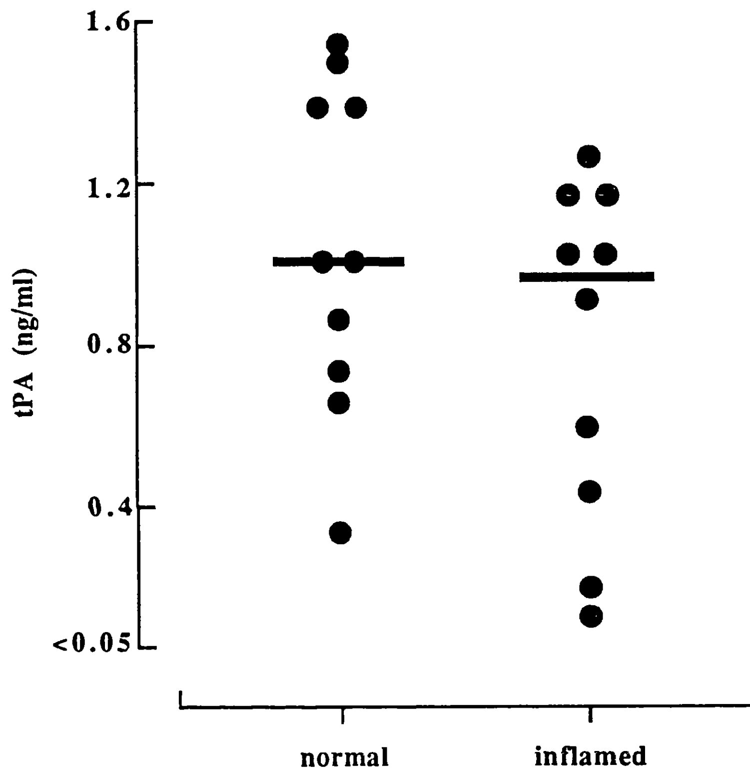

Figure 6.2 *Concentration of tissue plasminogen activator (tPA) in normal and inflamed human peritoneum (solid bar indicates median value)*

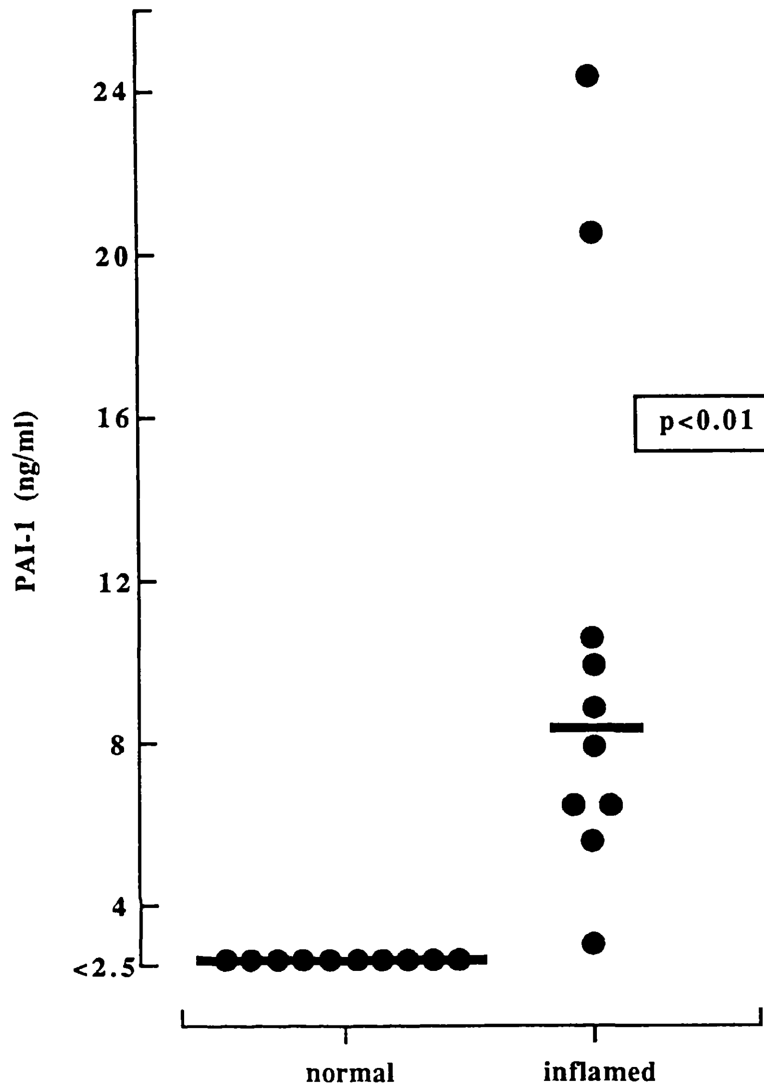

Figure 6.3 *Concentration of plasminogen activator inhibitor-1 (PAI-1) in normal and inflamed human peritoneum (solid bar indicates median value)*

6.4 Discussion

The finding of an elevated level of PAI-1 in inflamed peritoneum, which is not present in normal peritoneum, offers a plausible explanation for the loss of functional activity in injury. The immunoassay used to detect tPA and PAI-1 measures both bound and free forms of these molecules. The combination of results in this study suggests that the PAI-1 elaborated in inflammation has bound with tPA, the absolute level of which has remained unaltered, thus leading to loss of functional fibrinolytic activity.

One previous study has looked at the effect of inflammation in a serous cavity on PAI-1 concentrations (Agrenius et al 1989). These workers measured PAI-1 levels in pleural fluid after pleurodesis with quinacrine - the pleura is also lined by a single cell layer similar to mesothelium. PAI-1, which was present before commencing treatment, increased for 48 hours after quinacrine installation and this was related to the pleural inflammatory reaction. There was no significant change in α_2-antiplasmin levels. The authors concluded that overall fibrinolytic activity in pleura decreased with inflammation and this was related to the elaboration of PAI-1.

In the present study absence of detectable α_2-antiplasmin in either normal or inflamed peritoneal

tissue suggests that this enzyme has no important role in inhibiting fibrinolysis within the peritoneum. As α_2-antiplasmin is synthesised by the liver and secreted into the circulation its absence is not altogether surprising. However this raises the question of how plasmin generated by the action of tPA on plasminogen is cleared. There are three possibilities. Firstly: as tPA is fibrin-specific, all plasmin generated is limited to the fibrin surface and therefore there is little free plasmin available for inactivation. Secondly: α_2-antiplasmin is present only in the peritoneal fluid and inactivates only free plasmin within this fluid. Thirdly: any free plasmin present is reabsorbed across the peritoneum and inactivated systemically. The principal role of α_2-antiplasmin is to prevent systemic circulation of free plasmin so making the third possibility most likely. Additionally free plasmin within the peritoneal cavity is unlikely to be a major problem for two reasons: its substrate (fibrin) is not normally present and, if fibrin were present, it would be beneficial for this to be degraded.

The role of the other plasminogen activator inhibitors, PAI-2 and PAI-3, was not studied. PAI-2 inhibits uPA significantly better than tPA (Kruithof et al 1986) and, as uPA is almost certainly not present in peritoneum, it might only have a limited function even if detected. Similarly

PAI-3 is a comparatively poor inhibitor of tPA.

Despite these observations, recent studies on mesothelial cell cultures derived from human omentum found that PAI-2 was elaborated after stimulation with tumour necrosis factor (TNF) (van Hinsbergh et al 1990). PAI-2 has also been associated with peripheral blood leucocytes (Kopitar et al 1985) and as these will be present in an inflammatory exudate it may aid tPA inactivation in inflammation. Further studies on the relative roles of PAI-1 and PAI-2 in inflamed peritoneum *in vivo* would be of interest.

The source of PAI-1 has not been studied directly. By analogy with the vascular system, where PAI-1 is released by endothelial cells, the mesothelium is a likely source. Stimulation of endothelial cell cultures with lipopolysaccharide or TNF leads to increased PAI-1 production (Hanss & Collen 1987, Medina et al 1989) and this effect has also been shown *in vivo* (van Hinsbergh et al 1988). These cytokines may have the same effect on mesothelium in intra-peritoneal inflammation. The α-granules of platelets are also a rich source of PAI-1 (Erikson, Ginsberg & Loskutoff 1984) and a possible source in inflammation. The cDNA of PAI-1 has been isolated (Ny et al 1986) and gene probes could be used for cytological localisation in peritoneal tissue.

In this study inflammation of the assayed peritoneum was secondary to conditions in which operation was undertaken within 24 hours (acute appendicitis, perforated viscus). With experimental peritonitis in the rat (Chapter 4) the initial depression of PAA was followed by a rebound increase. If the same rebound phenomenon were to occur in human peritoneum this would suggest an increase in tPA levels occurring between 2 and 5 days. Such a response would enable inactivation of the PAI-1 released in response to the inflammation and return of functional activity enabling fibrinolysis to occur. This may have an important role as immediately after a peritoneal insult it is important for the injured site to be sealed (e.g. perforated viscus, leaking anastomosis) or to be localised (e.g. acute inflammatory focus). Once this has occurred it would be beneficial for fibrinolytic activity to return as formal healing occurs. The development of permanent adhesions would depend on the timing of return of functional fibrinolytic activity; that is the ability to lyse fibrinous deposits before organisation occurs. However, it must be remembered that the clinical situation is an evolving process of inflammation and differs from the animal studies where there was a single experimental insult.

These studies show that the peritoneum, as the vascular

system, has a mechanism for regulating fibrinolysis and that this balance is crucial to the healing process after injury. The fibrin-specific activator tPA is ideally suited to peritoneal fibrinolysis and PAI-1, elaborated by the peritoneum in inflammation, is the most efficient inhibitor of tPA. These findings suggest two possible approaches to the prevention of adhesions by the controlled enhancment of fibrinolysis. The continuing presence of tPA, even during inflammation, allows the use of anti-PAI-1 monoclonal antibody or other PAI-1 inhibitor to bind with the PAI-1 released and allow the innate tPA present to achieve plasminogen activation. An alternative approach is to provide exogenous tPA, in sufficient dosage, to inactivate the released PAI-1 and thus enhance peritoneal fibrinolysis.

CHAPTER 7

FIBRINOLYTIC ACTIVITY OF HUMAN PERITONEAL EXUDATE

7.1 Introduction

The presence of PAI-1 in inflamed human peritoneal tissue provides an explanation for the reduced functional fibrinolytic activity seen in peritoneal inflammation. This observation supports the hypothesis that reduced fibrinolytic activity is a common initiating pathway for adhesion formation secondary to intra-abdominal inflammation. However, in clinical practice, the commonest predisposing cause for adhesions is previous surgery; most frequently elective surgery for non-inflammatory conditions. In the studies described in the previous chapters peritoneal samples were taken early in the time course of surgery and thus do not reflect the influence of the surgical procedure on peritoneal fibrinolysis. Surgical procedures *per se* excite an inflammatory reaction secondary to mechanical trauma (Raftery 1973a). There may also be other factors such as peritoneal drying and contamination with blood and other fluids. Whilst it has been shown in animals that surgery leads to depressed fibrinolysis this is not true for human beings.

It is not ethical to re-operate on patients solely for the purpose of obtaining biopsies after surgery and early re-operation is rarely planned. However, in a number of procedures, drains are routinely employed and these provide a route to sample the peritoneal exudate that occurs after surgery. The aim of this study was to determine the

fibrinolytic parameters in the peritoneal exudate following elective abdominal surgery for non-inflammatory conditions.

7.2 Materials and methods

7.2.1 Patients

Five patients undergoing elective cholecystectomy for cholelithiasis were studied. All patients received 5 000 IU of heparin subcutaneously twice daily commencing preoperatively and continuing until they were fully mobile post-operatively as part of routine prophylaxis for deep vein thrombosis. Cholecystectomy was performed without complication in all patients; no patient required bile duct exploration and the gallbladder bed was not opposed with sutures. At the end of the procedure a polypropylene drain (Redivac, UK) was placed to the gallbladder bed and brought out through a separate incision.

The study was approvd by the local hospital ethical committee and all patients gave informed consent to inclusion in this study.

7.2.2 Samples

As soon as possible after the abdomen was opened a 5 ml sample of bile was obtained via direct aspiration of the gallbladder. At 24 hours post-operatively 5 ml of drain

fluid was collected and 5 ml of blood drawn without venous occlusion. As soon as the samples were obtained they were rapidly mixed with 10% v/v of 0.11 M buffered sodium citrate pH 4.5 in polypropylene tubes which were immediately transferred to an ice bath. The samples were centrifuged at 2 000 x *g* for 20 minutes at 4°C and 0.25 ml of the supernatant from each sample then stored at -80°C until assay.

7.2.3 Assays

The three samples from each patient were assayed for PAA by the fibrin plate method (Section 2.2.6); for tPA and PAI-1 by ELISA (Section 6.2.2). For the fibrin plate assay PAA, as determined from a standard curve, was expressed in IU of rtPA per ml of sample. The lower limit of detection for the assays was 0.025 IU/ml (PAA); 0.5 ng/ml (tPA) and; 2.5 ng/ml (PAI-1).

7.2.4 Statistical Analysis

Kruskal-Wallis non-parametric ANOVA was used for comparisons between groups.

7.3 Results

7.3.1 Patients

There were four women and one man, median age 46 years. All patients recovered uneventfully post-operatively. Median drainage was 35 ml and all drains were removed after the fluid sample had been drawn. Histological examination of the excised gallbladders confirmed chronic cholecystitis with no evidence of acute inflammation.

7.3.2 Fibrinolytic parameters

Tissue plasminogen activator was present in all the samples with no statistically significant difference between the three types of fluid (Figure 7.1). PAI-1 was not detectable in any of the bile specimens but was universally present in all serum and drain fluid samples (median PAI-1 of bile ≤ 2.5 ng/ml versus 24.99 ng/ml for serum and 32.65 ng/ml for drain fluid, $p<0.01$; Figure 7.2). Plasminogen activating activity was present in all bile samples whilst it was not detectable in either serum or drain fluid (median PAA of bile 0.34 IU/ml versus ≤0.025 IU/ml for serum and ≤0.025 IU/ml for drain fluid, $p<0.05$; Figure 7.3).

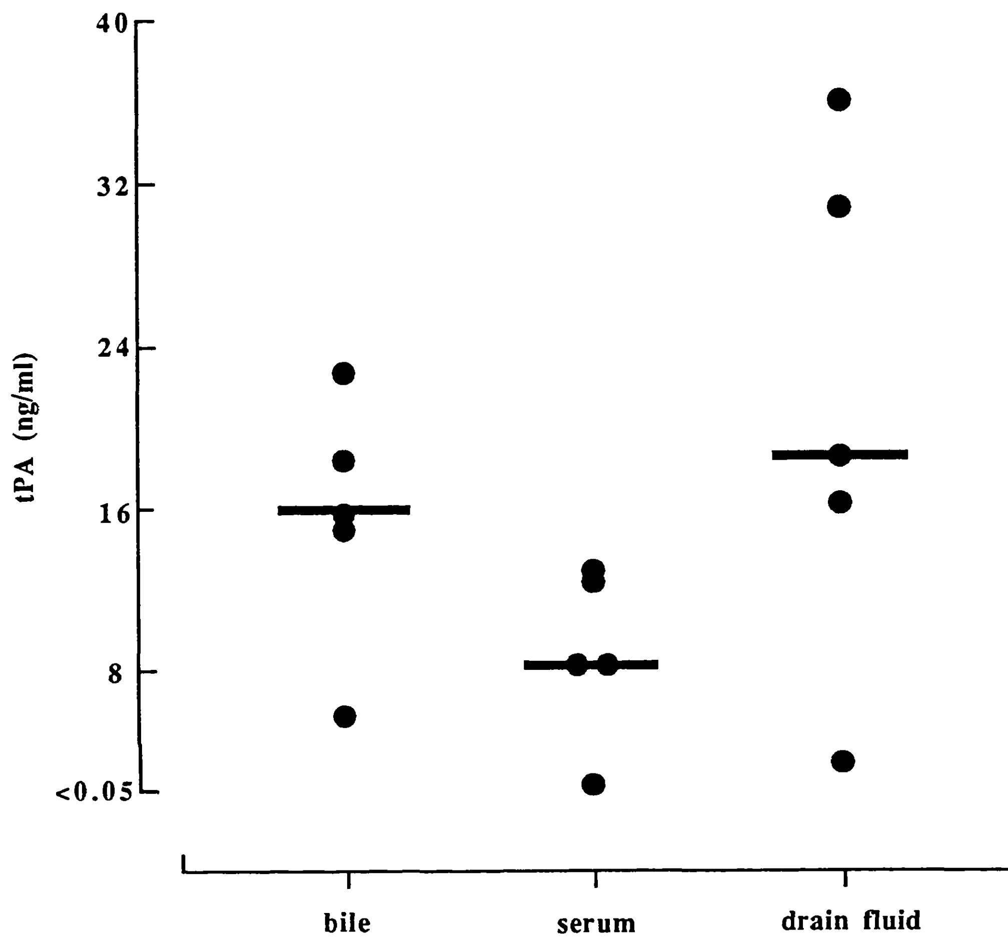

Figure 7.1 *Concentration of tissue plasminogen activator (tPA) in bile, serum and drain fluid (solid bars indicate median values)*

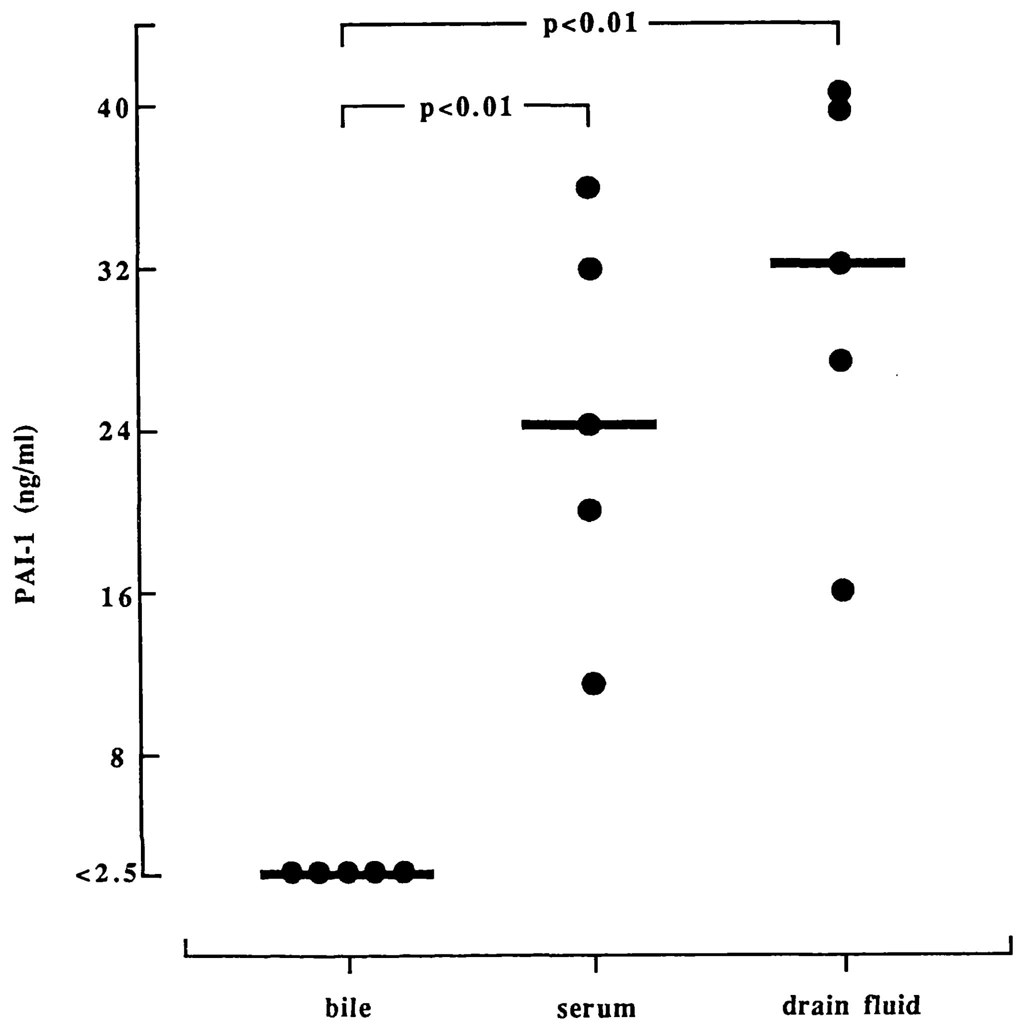

Figure 7.2 *Concentration of plasminogen activator inhibitor-1 (PAI-1) in bile, serum and drain fluid (solid bars indicate median values)*

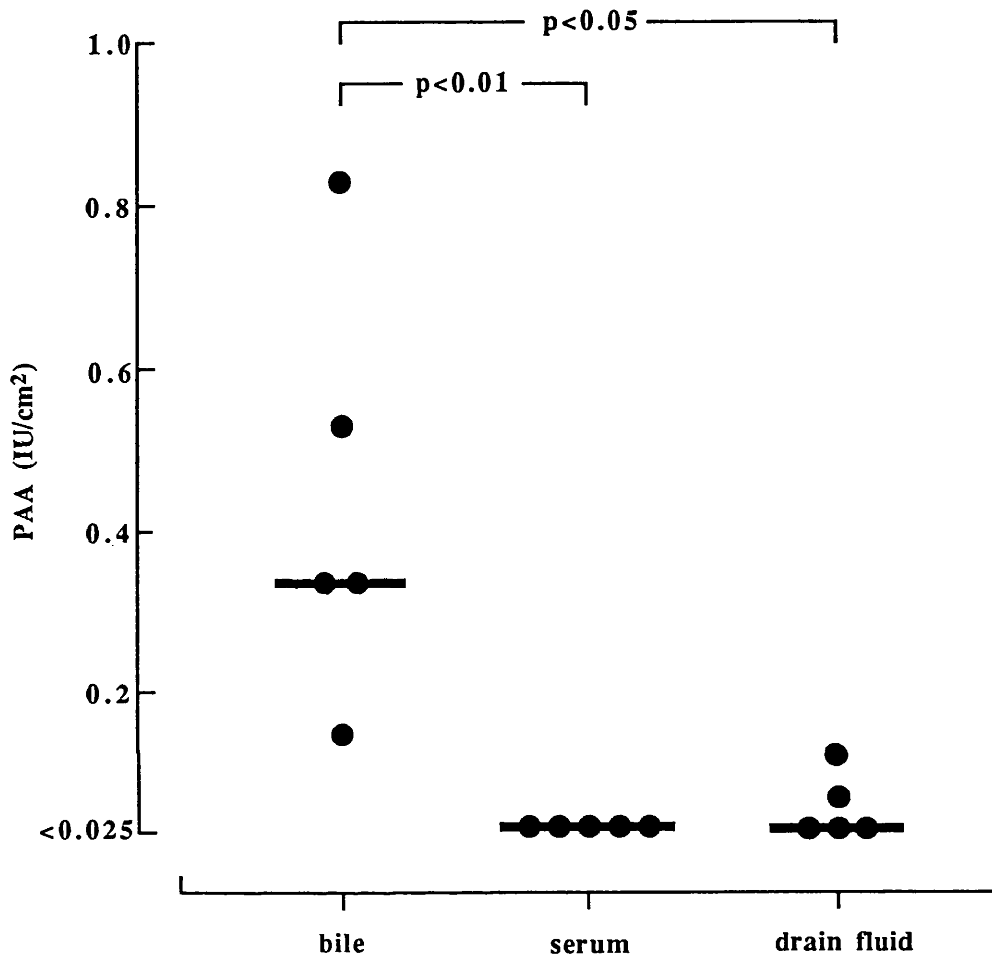

Figure 7.3 *Plasminogen activating activity (PAA) of bile, serum and drain fluid (solid bars indicate median values)*

7.4 Discussion

Drain fluid after cholecystectomy is produced by a combination of events: mechanical trauma of parietal and visceral peritoneal surfaces by operative manipulation; raw surfaces left by excision of the gallbladder; and the continuing inflammatory stimulus of the drain. Thus the fluid will contain mainly serous exudate, although it may also contain any bile that leaks from the gallbladder bed. Drain fluid had a high PAI-1 concentration with no plasminogen activating activity. As bile contained no detectable PAI-1 it is unlikely that bile contamination produced the reduction in PAA found in drain fluid. In the previous study (Chapter 6) PAI-1 was present only in inflamed peritoneum and it is therefore likely that the high PAI-1 levels found in drain fluid were derived from a peritoneal inflammatory exudate. The drain fluid sampled in this study had accummulated over 24 hours and thus reflects continuous sampling of what may be a dynamic situation. This to some extent, however, corresponds to the clinical situation when the peritoneal exudate remains in the peritoneal cavity until absorption. The results for drain fluid are very similar to those seen with inflamed peritoneum; i.e. neutralisation of tPA by PAI-1 resulting in the loss of fibrinolytic activity. The exudate will therefore fail to lyse any fibrinous adhesions that may form. This provides a mechanism for the formation of

adhesions to the gallbladder bed, which are familiar to all surgeons, and further supports the central role of peritoneal fibrinolysis in the pathogenesis of adhesion formation.

There have been few systematic studies of fibrinolytic activity of intraperitoneal fluid. In studies of ascites secondary to malignancy or cirrhosis, Baele, Rasquin & Barbier (1986) found high levels of fibrin degradation products which suggested that fibrinolytic activity was present in the ascitic fluid - although the presence of these products may have been related to the condition under study. Similarly Huber et al (1988) were able to demonstrate the presence of both tPA and PAI-1 in ascites fluid but did not measure plasminogen activating activity.

Conflicting results have been obtained for peritoneal fluid from women undergoing laparoscopy for non-inflammatory conditions. Plasminogen activating activity was not demonstrated using the fibrin plate method (Pattinson et al 1981) but with a different assay system PAA was detected (Batzofin et al 1985). A thin film of peritoneal fluid is present in the normal state and it is possible that this contains PAA which plays a role in preventing adhesion of peritoneal surfaces. Further studies are required of peritoneal fluid from patients without inflammatory foci to resolve this question although the volumes present are usually very small.

In continuous ambulatory peritoneal dialysis (CAPD) fluid is instilled into the peritoneal cavity and exchanges across the peritoneal membrane. This treatment is a valuable therapeutic option in the management of renal failure in both adults and children. Peritonitis is a major complication of such treatment with an average of 2 episodes per patient year (Chan et al 1981). Infection is a major cause of fibrin production during CAPD and can result in catheter blockage, peritoneal sclerosis and small bowel obstruction from adhesions (Gandhi et al 1980, Schmidt & Blumenkrantz 1981). Examination of peritoneal fluid from patients undergoing CAPD for fibrinolytic parameters would be of interest. If this fluid showed the same features of depressed PAA, as seen in patients post-cholecystectomy, the possibility exists of adding fibrin specific agents (such as tPA) to dialysis fluid to reduce these severe complications. Urokinase has already been used with some success in a small number of patients (Benevent et al 1985, Pickering et al 1988).

The presence of PAA in bile has not previously been described. However the bile asssayed in this study may not be entirely normal as it was obtained from patients with cholelithiasis. The absence of PAI-1 and presence of tPA provides this bile with plasminogen activating activity. A physiological role for this activity is not clear but it would certainly enable lysis of any fibrin that formed in

bile and thus help to maintain its fluidity which may be of importance in gallbladder emptying. It is well recognised that bile leaks following biliary surgery are often slow to close and this may be related to rapid lysis of any fibrinous plugs that form. It would be of interest to assay gallbladder mucosa for fibrinolytic system enzymes and to examine bile from patients with acute cholecystitis.

The concentrations of tPA and PAI-1 found in post-operative serum are comparable to other studies (Mellbring et al 1984, Páramo, Alfaro & Rocha 1985). Although not examined in the present study, it is known that PAI-1 levels increase in the immediate post-operative period and this has been linked with an increased susceptibility to venous thrombosis (Gomez et al 1987).

This study has not shown for how long the post-operative peritoneal exudate lacks fibrinolytic activity but this would not be easy to determine. Sampling from an external drain beyond 24 hours is likely to give erroneous results as the presence of a drain would provide a continuing inflammatory stimulus to the surrounding peritoneal tissues. An alternative approach would be by percutaneous aspiration of peritoneal fluid but this may be hazardous and ethically unacceptable.

In summary, this study has shown that the peritoneal exudate 24 hours following cholecystectomy has no plasminogen activating activity. The lysis of fibrinous

adhesions at this time is prevented, allowing organisation of fibrin and the development of permanent adhesions. Thus any measures designed to enhance fibrinolysis and reduce adhesion formation would probably have to be effective for at least 24 hours post-operatively.

CHAPTER 8

REDUCTION OF ADHESIONS WITH RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR

8.1 Introduction

Strategies to prevent adhesion formation can be based on the identification of the physiological mediators of peritoneal fibrinolysis. As described in Chapter 6, tPA is the major plasminogen activator in human peritoneum and confers plasminogen activating activity whilst PAI-1 is found to be present after peritoneal injury and antagonises the action of tPA. From these findings, there are two possible mechanisms to enhance fibrinolytic activity. Firstly, a PAI-1 inhibitor such as anti-PAI-1 monoclonal antibody could be used to inactivate the PAI-1 present following peritoneal injury and allow the existing peritoneal tPA to lyse any fibrinous adhesions that may form. Alternatively, tPA could be delivered locally in excess to overcome the effect of the PAI-1 released in injury. Any excess tPA will then be able to degrade free fibrin present and potentially limit the formation of adhesions.

PAI-1 inhibitors are not yet available but tPA has been produced via recombinant DNA technology (rtPA). It was isolated initially from human tissues (Rijken et al 1979) and has been produced in useful quantities from a human melanoma cell line (Collen et al 1982, Kluft et al 1983). Following this development the efficacy of systemically delivered rtPA to lyse clot in patients with acute coronary atery thrombosis has been shown in several clinical trials

(Collen et al 1984, Verstraete et al 1985, Wilcox et al 1988).

The studies of experimental peritonitis in animals (Chapter 4) indicate that the period of reduced PAA of the peritoneum after injury lasts at least 24 hours. Any rtPA delivered locally would therefore need to be present in the peritoneal cavity and remain active for at least this length of time. In the trials of coronary aterial occlusion rtPA was delivered as an aqueous solution. However, within the peritoneal cavity, such solutions are absorbed within a few hours and do not persist beyond 24 hours (Ellis 1971).

The present study was performed in two parts. Firstly, the incorporation of rtPA in an inert viscous gel with studies to determine that biological activity persisted for 24 hours. Secondly, the intraperitoneal application of rtPA in a rat adhesion model to determine its efficacy in reducing adhesions.

8.2 Study I: Incorporation of rtPA within a carrier gel

8.2.1 Introduction

A gel carrier for a pharmacologically active agent should, if possible, have the following characteristics: provide constant delivery of the agent; not affect the

efficacy of the delivered agent; and in itself be inert. Methylcellulose is a hydrophilic colloid which binds loosely with water and is widely used as an inert carrier for topical application of many drugs. It is not associated with any adverse or irritant effects when used internally (Homsey, Stanley & King 1973). The viscosity of methylcellulose gel can be altered depending on the proportion of lyophilised methylcellulose used in reconstitution with an aqueous solution.

8.2.2 Materials and methods

Initially, 5 ml aliquots of aqueous recombinant tissue plasminogen activator (rtPA, Boehringer Ingelheim, Bracknell, UK) were prepared at a concentration of 1 mg/ml. These aliqots were then used to reconstitute 1.5% methylcellulose powder (Sigma Chemical Co., Poole, UK) and produce, in duplicate, rtPA-gels of three viscosities (1500, 2250 and 3000 cps). The rtPA-gels were stored at 4°C.

One day later the rtPA-gel solutions were transferred to a portion of cylindrical dialysis tubing with pores of 300 kD (Spectrapore, USA). The ends of the dialysis tubing were sealed with plastic clips and the tubing placed in a glass beaker containing 50 ml of 0.9% saline at 37°C. At 2 hours 1 ml of the saline surrounding the dialysis tubing was removed and stored at -80°C. The remaining saline was

discarded and replaced with 50 ml of fresh saline. This procedure was repeated at 2 hourly intervals for 24 hours. To determine the biological activity of the diffusate from the dialysis tubing, plasminogen activating activity (PAA) for each of the samples was determined by the fibrin plate method previously described. Twenty µl of each sample was placed on fibrin plates, incubated at 37°C and read at 24 hours. To act as controls 20 µl aliquots of 0.9% saline were also placed on fibrin plates and read at the same time.

8.2.3 Results

Plates incubated with samples of diffusate showed complete lysis of the entire fibrin plate at all time intervals and for all viscosities of rtPA-gel. For this assay this represented a PAA of ≥4.25 IU/ml. No lysis was observed with the control saline samples.

The physical appearance of the three viscosities ranged from nearly liquid (1500 cps) to semi-solid (3000 cps). The gel of viscosity 2250 cps had a similar consistency to commercially available lubricating jelly.

8.2.4 Discussion

This pilot study indicated that it was possible to incorporate rtPA within a viscous gel while maintaining PAA. Factors affecting the diffusion of rtPA from the gel

in this model include the diffusion gradient, the size of the dialysis tubing pores and binding of rtPA with methylcellulose. The latter is not known but the diffusion gradient was totally in favour of rtPA diffusion and the dialysis pores of 300 kd should easily have allowed passage of rtPA which has a molecular weight of 72 kD. The results from this admittedly somewhat crude diffusion study indicate that rtPA was still present within the methylcellulose at 24 hours and at this time it still retained a PAA in excess of 4.25 IU/ml. Additionally, this activity was not affected by storage for 24 hours at 4°C. The physical appearance of the rtPA-gel of viscosity 2250 cps was the most suitable for ease of use *in vivo*.

8.3 **Study II: Effect of rtPA-gel on adhesion formation**

8.3.1 Introduction

To study the effect of rtPA on adhesion formation a reproducible and quantifiable animal model of adhesion formation is required. The method of ligated buttons of rat peritoneum described in Chapter 4 allows the presence of adhesions to be accurately and objectively quantified: an adhesion is either present or absent to a ligated button. With six buttons per animal this gives a possible

adhesion score of 0 - 6 for each animal. This model is also simple and reproducible producing a median adhesion score of five from one day onwards (Figure 4.8).

8.3.2 Materials and methods

Twelve aliquots (5 ml) of rtPA incorporated in methylcellulose gel were made up at a concentration of 1 mg/ml rtPA and at a viscosity of 2250 cps buffered to pH 7.4. Also twelve 5 ml aliquots of methylcellulose gel (2250 cps) alone were prepared. The 24 aliquots were drawn up into individual syringes, numbered randomly from 1 - 24 and stored at 4°C. The contents of each numbered syringe was known only by a third party.

Twenty four hours later peritoneal buttons were formed in 36 male Wistar rats weighing 250-300g. Under ether/air anaesthesia the abdomen was opened in the mid-line. A button of parietal peritoneum adjacent to the paracolic gutter was picked up in fine forceps and the base ligated with 3/0 chromic catgut (Ethicon Ltd, Edinburgh, UK). Three buttons were created along each paracolic gutter. At the completion of the laparotomy each animal was randomised to one of three groups: (A) no treatment control; (B) gel; (C) rtPA-gel. Randomisation was performed by a third party with the operator unaware of each group. The appropriate numbered syringe, the contents of which were unknown to the operator, was then instilled intraperitoneally in animals

in groups (B) and (C). The abdomen was closed in two layers; continuous 3/0 PDS (Ethicon Ltd, Edinburgh, UK) to the muscle layer and continuous 3/0 Nurolon (Ethicon Ltd, Edinburgh, UK) to the skin. The skin was then sprayed with povidone-iodine. Animals were returned to their cages, recovered and allowed food and water *ad libitum*.

One week later all animals underwent a second laparotomy. Adhesions were scored by the operator unaware of the treatment group to which each animal belonged: the code being held by the assistant. An adhesion score of 0 - 6 was given to each animal.

8.3.3 Results

The adhesion score for each animal is shown in Figure 8.1. For the no treatment control animals the median number of adhesions per animal was 5 (range 3 - 6), for the gel alone treated group 2.5 (range 1 - 5) and for the rtPA-gel treated animals 1.5 (range 0 - 3). These differences were statistically significant (control vs. gel alone, $p<0.01$; gel alone vs. rtPA-gel, $p<0.05$; control vs. rtPA-gel, $p<0.001$; Kruskal-Wallis ANOVA).

No adverse effect was noted in any animal - in particular laparotomy wounds were soundly healed and there was no evidence of haemorrhage. At the second laparotomy there was neither residual evidence of the gel nor any excess free peritoneal fluid.

Figure 8.1 *Adhesion scores for control, gel alone and rtPA-gel groups (solid bars indicate median score)*

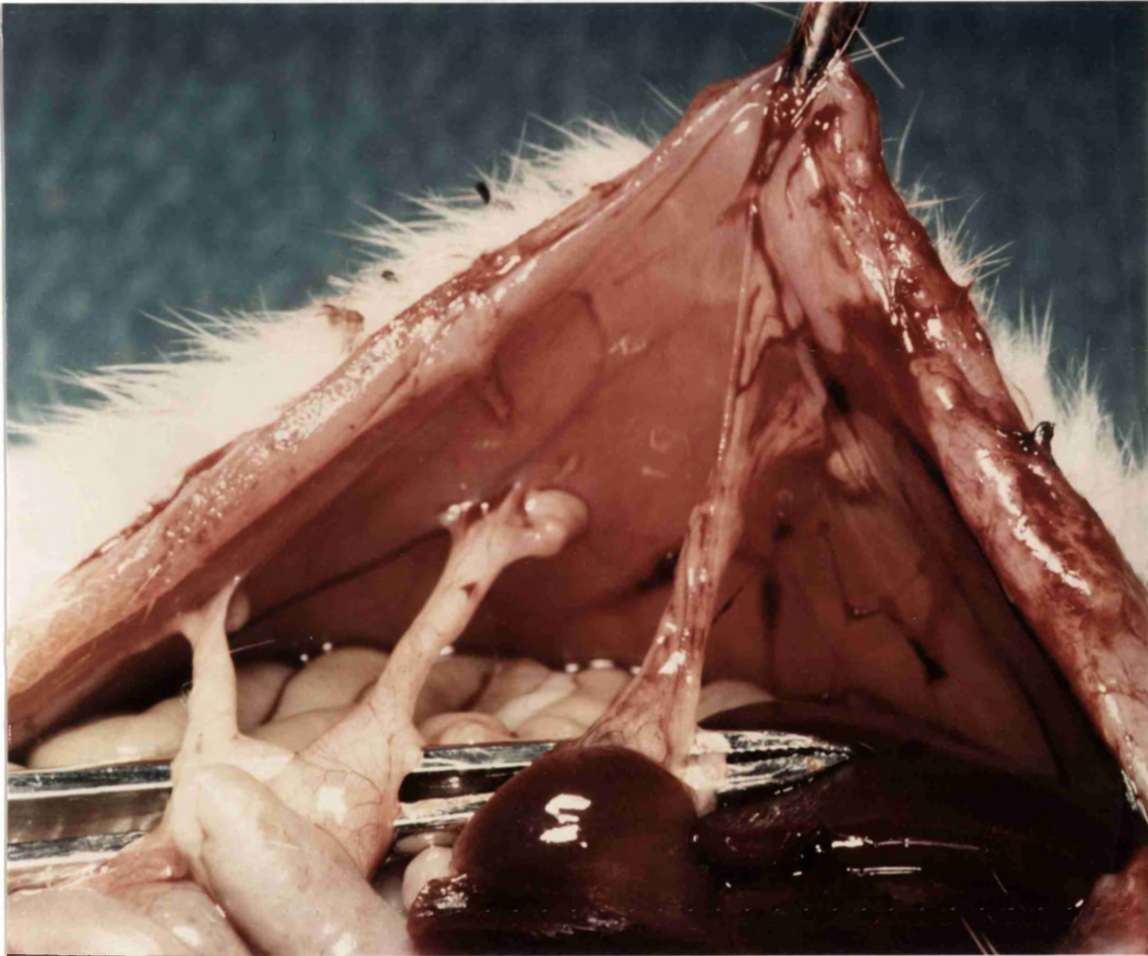

Figure 8.2 *Control animal showing omental adhesions to ischaemic buttons on lateral peritoneal wall*

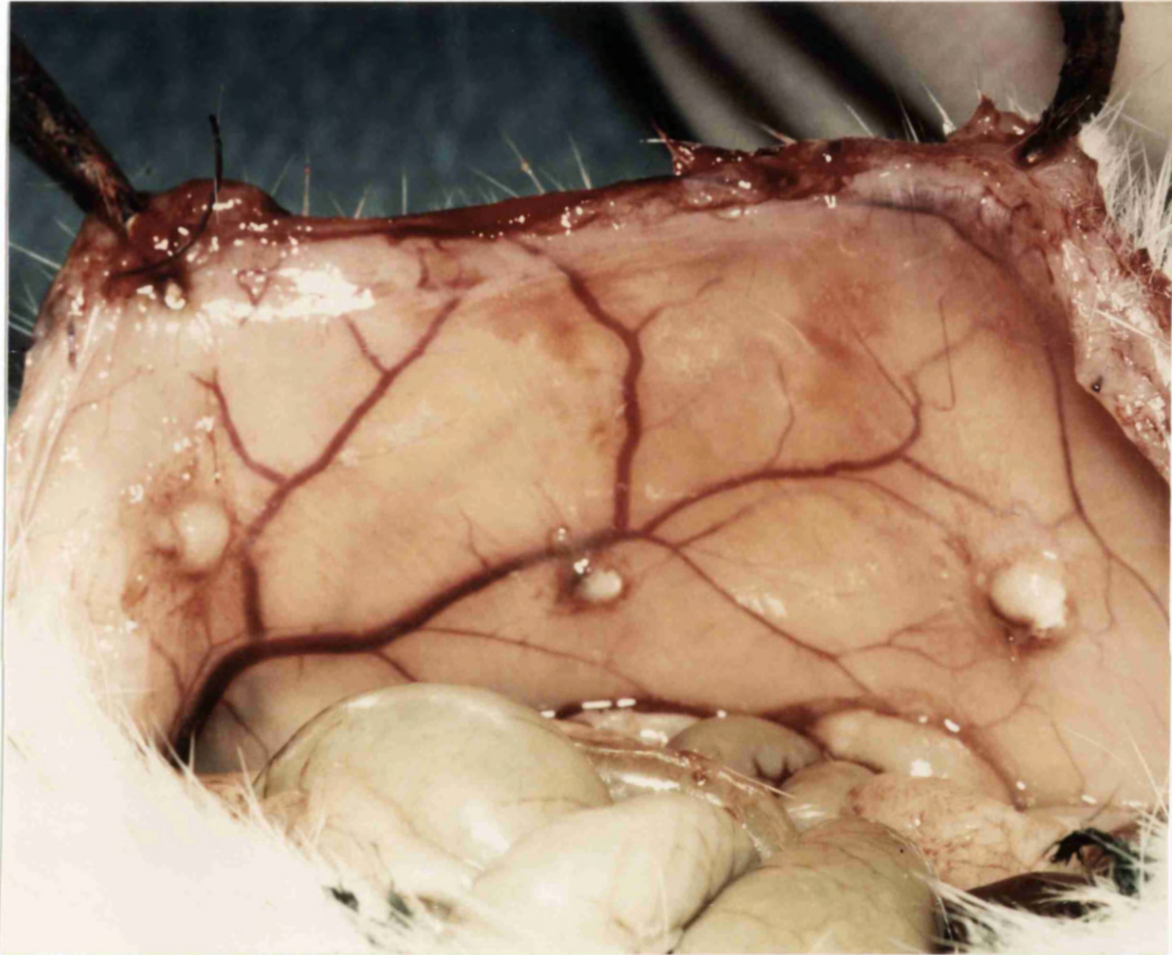

Figure 8.3 *rtPA-gel treated animal showing absence of adhesions - buttons are easily visible on lateral peritoneal wall*

8.4 Discussion

Although intraperitoneal rtPA-gel significantly reduced adhesion formation in this model it is of interest that the inert carrier, methylcellulose, also reduced adhesions compared with no treatment control animals. Sodium carboxymethylcellulose has also been used in other studies and shown to reduce intra-abdominal adhesions in the rat (Elkins et al 1984a, Elkins et al 1984b). Fredericks et al (1986) examined the effect of varying doses of sodium carboxymethylcellulose. These ranged from 1-3% solutions although the precise viscosity was not stated. An abrasion injury to rabbit uterine horn was used to produce adhesions and these were scored subjectively from 0-4. Adhesions were reduced by all solutions of sodium carboxymethylcellulose but were not entirely prevented. The effect was found to be dose dependent. These workers suggested that as carboxymethylcellulose is physiologically inert and has no effect on fibrinolysis the agent acted mechanically as both a barrier and lubricant.

Fibrinolytic agents have also been investigated previously. Using a peritoneal abrasion model in dogs or rabbits streptokinase solutions have been shown to reduce the incidence of adhesions (Wright et al 1950, Luttwak, Feldman & Neuman 1954, Sherry, Callaway & Freiberg 1955). However, with an ischemic button model in rats, no reduction in adhesion formation was found when

streptokinase was given for up to 72 hours (James, Ellis & Hugh (1965). Streptokinase-activated plasminogen has been found to reduce adhesion formation when given as a single intraperitoneal dose (Luttwak, Behar & Saltz 1957, Knightly, Agostino & Cliffton 1962), but these results were not corroborated by other studies (Bryant 1963, Jewett et al 1964). Differences in animal models, adhesion scoring and reagent dosage preclude direct comparison of these studies, though the use of aqueous solutions which are rapidly absorbed across the peritoneum may have contributed to the lack of efficacy in some reports. More recently urokinase has been found to be ineffective in adhesion prevention when given either intraperitoneally or intravenously (Rivkind, Lieberman & Durst 1985).

Other workers have also incorporated rtPA in a carrier gel and examined the effect on adhesion models. Menzies and Ellis (1989) instilled an rtPA gel intraperitoneally in the rabbit after surgically dividing adhesions that had already formed. rtPA-gel significantly reduced adhesion reformation compared to gel alone. Details of the dose of rtPA used or the volume or type of gel were not given in the paper, but is understood to be 3 mg of rtPA in a hyaluronic acid base (D. Menzies, personal communication). Hyaluronidase is also a weak plasminogen activator and, if present, may have contributed to the fibrinolytic effect observed.

Doody (1989) used rtPA combined with an inert gel, the nature of which was not stated, in a rabbit model of uterine horn abrasion. Varying doses of rtPA were employed from 0.3 mg to 12.5 mg. As in the present study gel alone significantly reduced adhesion formation, using a subjective scoring system, and rtPA-gel added significantly to this effect though only in doses greater than 1.25 mg. Additionally, no alteration in plasma fibrinogen or tPA levels was detected in blood samples taken at 2 hours.

A further intraperitoneal use of rtPA, investigated in rats, is the prevention of intra-abdominal abscess formation. Intraperitoneal inoculation of rats with fibrin clots infected with *Bacteroides fragilis* resulted in abscesses in all animals - these were completely prevented by local administration of aqueous rtPA (Rosenthal et al 1988). In a further study, (Rotstein & Rao 1988), intraperitoneal fibrin clots infected with a *B. fragilis and Escherischia coli* combination produced abscesses in all animals with a mortality of 44%. Intraperitoneal rtPA prevented absess formation but mortality increased to 81% with a three fold increase in the level of blood borne *E. coli.* The use of concomitant parenteral antibiotics in the rtPA treated group prevented all deaths. Similarly the delayed administration of local rtPA has been found to reduce abscess formation (McRitchie, Cummings & Rotstein 1989).

Recombinant tPA has also been used after intraocular surgery where subsequent fibrin formation may lead to blindness. The aqueous humour of patients undergoing elective cataract surgery is known to contain tPA (Tripathi et al 1988). Local delivery of exogenous rtPA after experimental fibrin formation in the anterior chamber of rabbits led to rapid resolution of fibrin clots with no adverse effects (Snyder, Lambrou & Williams 1987, Johnson, Olsen & Hernandez 1988).

These studies all indicate that rtPA delivered locally to body cavities can limit fibrin formation where this may be detrimental. In the present study of intra-abdominal adhesions, rtPA in a slow release gel was shown to significantly reduce adhesion formation. The viscous gel alone led to some reduction in adhesions but a further reduction occurred with the incorporation of rtPA. It is likely that two mechanisms are at work: the methylcellulose acts as a lubricating barrier and rtPA lyses any fibrinous adhesions that form. However, in these models it is important to consider the volume of gel instilled. In the current study this was 5 ml which equates to 20 ml/kg and in the study of Fredericks et al (1986), using gel alone, 7 ml/kg. If this volume was extrapolated to clinical use in man this would equate to 500 - 1500 ml in a 70 kg adult. Further studies on reducing this volume of gel whilst retaining the concentration of rtPA would be of interest to

minimise possible problems with the instillation of such large volumes.

The dose of rtPA in the present study was 5 mg and for the other study in which dosage was stated (Doody 1989) ranged up to 12.5 mg. These are very high doses compared with the concentration of tPA found in plasma, but such doses do appear to be required as no beneficial effect was seen in the study of Doody below a dose of 1.25 mg compared with carrier gel controls. No adverse effect of such a high dose was seen in either study. The recombinant molecule used in these studies is derived from human tPA and this might reduce its efficacy in animal models. There are slight species differences between human and rat tPA (Kjaeldgaard, Larsson & Astedt 1984). These may significantly affect the binding of human rtPA with native rat plasminogen and fibrin which is essential for efficient fibrinolysis. The efficacy of rtPA in human beings may thus be greater.

The use of rtPA in a slow release gel as a method of adhesion prevention or prophylaxis is very attractive. Because the action of tPA is localized to fibrin deposits, fibrinolytic activity is limited to this site which prevents indiscriminate fibrinolysis. Further studies are required to reduce the volume of carrier gel and identify the optimum dose of rtPA. Also serial measurement of systemic fibrinolytic parameters and wound healing are

required to fully determine if there are any adverse effects of this potential new treatment.

SUMMARY AND FURTHER INVESTIGATIONS

The studies described in this thesis have indicated that: (1) There is a close relationship between the plasminogen activating activity of the peritoneum and subsequent adhesion formation. (2) PAA was identified in both animal and human peritoneum and this activity was attenuated by inflammation or ischaemia: both are conditions associated with adhesion formation. (3) After standardised peritoneal injuries, PAA was initially depressed, then increased above normal levels and later returned to basal levels at two weeks. (4) In human peritoneum, the mediator of PAA was identified as tPA and depression of this activity, seen in inflammation, was related to the presence of PAI-1. (5) A similar relationship between these mediators was seen in post-operative peritoneal exudate. (6) Using an animal model of adhesion formation an inert gel was found to reduce adhesion formation and rtPA significantly increased this effect.

These studies, though, have also identified areas in which further work is required to clarify the relationship between peritoneal fibrinolysis and adhesion formation:

(1) The finding of depressed PAA immediately after three distinct peritoneal injuries in rats provides strong evidence that loss of the biological initiator of fibrinolytic activity prevents the lysis of fibrin deposits

in the peritoneal cavity in these conditions. However PAA returns at 24 hours yet adhesions formed at this time are not lysed. This is despite collagen incorporation not being apparent until three days. It is possible that the fibrin formed by 24 hours is cross-linked in such a way as to protect it from plasmin degradation. Alternatively plasminogen activators may not be able to penetrate the fibrinous adhesions or may not delivered to the adhesion at this stage. It is also possible that other unidentified factors play a role in adhesion formation.

(2) Further studies of the changes in PAA in the first four days after peritoneal injury are indicated, as are measurements of PAA in peritoneal fluid or early adhesions. In performing such studies the selection of an appropriate animal model is important as a wide variation in measured PAA occurs, probably due to species differences in substrate reactivity. The relative changes of the principal molecular mediators of PAA in such animal models of peritonitis would also be of interest but as yet no reliable method of assay is available.

(3) In human peritoneum these mediators can be detected reliably. Further studies of the other plasminogen activator inhibitors (two and three) in normal and inflamed tissue are required to establish whether they have a role

in affecting PAA. Whilst changes in such mediators over an extended period of time would be difficult to determine in human peritoneum *in vivo*, it would be possible to sample peritoneum at the start and end of an operative procedure. Similarly comparison of non-inflamed peritoneal fluid with inflammatory peritoneal exudate would be of interest. The use of cell culture of human peritoneum would allow the effect of individual inflammatory mediators on fibrinolytic parameters to be examined *in vitro*.

(4) The precise site of plasminogen activator production has not been established but the recent availability of cDNA probes for these molecules may make this possible by *in situ* hybridisation techniques.

(5) Other serous membranes, such as the pleura and pericardium, are complicated by formation of adhesions. The role of fibrinolytic enzymes in these mesothelial linings may be similar and requires investigation.

(6) The reduction of adhesions by rtPA-gel in an animal model is encouraging. Further work is required to determine the optimum volume and dose of such a preparation. Systemic absorption needs to be measured and the effect on wound and anastomotic healing determined. Because of species differences in plasminogen activators,

rtPA might be expected to achieve greater efficacy in human beings. Adhesions also prove troublesome in other body cavities and exogenously delivered plasminogen activators may be of benefit in these situations, e.g. pleural, pericardial and synovial cavities and with prosthetic implants.

(8) The studies described in this thesis form the basis for further studies aimed at the application of this knowledge of peritoneal fibrinolysis to clinical use. Intra-abdominal adhesions only cause complications in a small proportion of patients after abdominal surgery although the actual numbers are high and the problems may be considerable. One can envisage the use of intraperitoneal rtPA after surgical division of adhesions that have caused complications or as primary prophylaxis, for example to prevent adhesions to the undersurface of an abdominal incision. Methods would need to be developed for assessing the efficacy of such treatment. Currently this would involve second-look surgery (laparotomy or laparoscopy) or long-term randomised studies to measure re-obstruction rates. However it may be possible to develop non-invasive methods of assessment.

In conclusion, this thesis raises many new questions as more is discovered about peritoneal fibrinolysis.

Nevertheless, application of this current knowledge, combined with future work, leads to the possibility of developing a physiological approach to adhesion prophylaxis - a problem that has taxed the surgeon for much of the twentieth century.

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APPENDIX

APPENDIX CHAPTER 2

Data for standard curve shown in Figure 2.2

rtPA concn (IU/ml)	rtPA in 20 µl aliquot (IU)	$\log_{10}$ rtPA (IU)	zone of lysis (cm)
0.0425	0.00085	-3.0706	0.90
0.085	0.0017	-2.7696	0.95
0.425	0.0085	-2.0706	1.25
0.85	0.017	-1.7696	1.45
4.25	0.085	-1.0706	1.70
8.5	0.17	-0.7696	1.90

Fibrinolytic activity of animal peritoneum

I: Rat peritoneum

animal	homogenate (IU/cm^2)	gel disc (IU/cm^2)	whole tissue (IU/cm^2)
1	0.17	0.037	0.037
	0.13	0.037	0.037
2	0.29	0.037	0.037
	0.17	0.05	0.02
3	0.1	0.037	0.011
	0.13	0.02	0.011
4	0.29	0.037	≤0.01
	0.17	0.02	≤0.01
5	0.29	0.02	0.011
	0.13	0.02	0.011
6	0.1	0.02	≤0.01
	0.17	0.02	≤0.01
median	0.18	0.029	0.011

II: Rabbit peritoneum

animal	homogenate (IU/cm^2)	gel disc (IU/cm^2)	whole tissue (IU/cm^2)
1	≤0.006	≤0.01	≤0.01
	≤0.006	≤0.01	≤0.01
2	≤0.006	≤0.01	≤0.01
	≤0.006	≤0.01	≤0.01
3	0.019	≤0.01	≤0.01
	0.013	≤0.01	≤0.01
4	0.019	≤0.01	≤0.01
	0.019	≤0.01	≤0.01
5	≤0.006	≤0.01	≤0.01
	≤0.006	≤0.01	≤0.01
6	0.019	≤0.01	≤0.01
	0.025	≤0.01	≤0.01
median	0.019	≤0.01	≤0.01

III: Guinea-pig peritoneum

animal	homogenate (IU/cm^2)	gel disc (IU/cm^2)	whole tissue (IU/cm^2)
1	0.134	≤0.01	≤0.01
	0.134	≤0.01	≤0.01
2	0.07	≤0.01	≤0.01
	0.05	≤0.01	≤0.01
3	0.07	≤0.01	≤0.01
	0.096	≤0.01	≤0.01
4	0.134	≤0.01	≤0.01
	0.134	≤0.01	≤0.01
5	0.05	≤0.01	≤0.01
	0.07	≤0.01	≤0.01
6	0.036	≤0.01	≤0.01
	0.07	≤0.01	≤0.01
median	0.072	≤0.01	≤0.01

Fibrinolytic activity detected with pre-heated fibrin plates

	animal species		
	rat (IU/cm^2)	rabbit (IU/cm^2)	guinea-pig (IU/cm^2)
biopsy number			
1	≤0.006	≤0.006	≤0.006
2	≤0.006	≤0.006	≤0.006
3	≤0.006	≤0.006	≤0.006
4	≤0.006	≤0.006	≤0.006
5	≤0.006	≤0.006	≤0.006
6	≤0.006	≤0.006	≤0.006

Validity of fibrin plate assay

Intra-Assay Variation

fibrin plate	rtPA (IU in 20 µl sample)				
	1	2	3	4	5
1	0.0019	0.0085	0.0150	0.0850	0.1564
2	0.0017	0.0102	0.0145	0.0680	0.1496
3	0.0018	0.0085	0.0181	0.0884	0.1564
4	0.0017	0.0085	0.0170	0.0680	0.1972
5	0.0015	0.0098	0.0168	0.0816	0.1360
CV	7.9%	9.1%	9.3%	12.3%	14.4%

Inter-Assay Variation

fibrin plate	rtPA (IU in 20 µl sample)							
	1	2	3	4	5	6	7	CV
1	0.018	0.016	0.020	0.014	0.017	0.016	0.018	13.9%
2	0.017	0.017	0.017	0.020	0.015	0.017	0.017	7.1%
3	0.014	0.017	0.017	0.018	0.020	0.016	0.018	12.5%
4	0.018	0.016	0.017	0.016	0.020	0.018	0.017	6.7%
5	0.016	0.014	0.018	0.016	0.014	0.019	0.018	12.8%

Plasminogen activating activity (IU/cm^2) of normal and inflamed human parietal peritoneum and omental visceral peritoneum

biopsy	parietal peritoneum		visceral peritoneum	
	normal	inflamed	normal	inflamed
1	3.0	0.106	9.12	0.06
2	9.12	0.06	5.23	0.106
3	3.0	0.08	12.05	0.08
4	5.23	0.106	3.96	0.08
5	15.92	0.08	2.27	0.14
6	9.12	0.08	0.57	0.06
median	7.18	0.08	4.59	0.08

APPENDIX CHAPTER 4

Plasminogen activating activity (IU/cm²)
for experimental peritonitis groups.

I: Sham Laparotomy

time	animal	biopsy number 1	2	3	4
6 hours	1	0.256	0.256	0.363	0.112
	2	0.112	0.112	0.256	0.27
	3	0.112	0.256	0.112	0.112
	4	0.112	0.112	0.063	0.112
	5	0.084	0.112	0.084	0.112
12 hours	1	0.112	0.112	0.112	0.112
	2	0.112	0.256	0.112	0.256
	3	0.084	0.112	0.112	0.112
	4	0.112	0.063	0.112	0.256
	5	0.084	0.15	0.112	0.15
1 day	1	0.256	0.256	0.256	0.652
	2	0.112	0.15	0.363	0.27
	3	0.486	0.652	0.256	0.112
	4	0.256	0.363	0.112	0.27
	5	0.27	0.15	0.652	0.363
4 days	1	0.363	0.256	0.27	0.486
	2	0.27	0.256	0.363	0.363
	3	0.486	0.256	0.363	0.363
	4	0.27	0.27	0.363	0.363
	5	0.15	0.27	0.084	0.112
7 days	1	0.286	0.112	0.27	0.256
	2	0.112	0.112	0.112	0.363
	3	0.063	0.363	0.27	0.15
	4	0.112	0.112	0.112	0.27
	5	0.035	0.084	0.063	0.063
10 days	1	0.112	0.363	0.112	0.256
	2	0.486	0.15	0.363	0.486
	3	0.256	0.363	0.27	0.256
	4	0.27	0.256	0.256	0.256
	5	0.112	0.112	0.27	
14 days	1	0.256	0.486	0.363	0.256
	2	0.256	0.256	0.27	0.363
	3	0.112	0.084	0.256	0.084
	4	0.112	0.112	0.27	0.112
	5	0.112	0.084	0.256	0.063

II: Bacterial Peritonitis

time	animal	biopsy number 1	2	3	4
6 hours	1	0.043	0.043	0.043	0.043
	2	0.034	0.043	0.024	0.024
	3	0.043	0.043	0.024	0.043
	4	0.024	0.032	0.043	0.073
	5	≤0.014	≤0.014	0.024	≤0.014
12 hours	1	0.024	≤0.014	≤0.014	≤0.014
	2	0.024	0.024	0.024	0.024
	3	0.024	0.043	0.043	0.024
	4	0.032	0.024	0.024	0.024
	5	0.024	≤0.014	0.024	≤0.014
1 day	1	0.26	0.50	0.36	0.36
	2	0.19	0.50	0.69	0.19
	3	0.26	0.36	0.26	0.36
	4	0.36	0.36	0.36	0.19
	5	0.26	0.36	0.50	0.36
4 days	1	1.32	0.36	0.69	0.69
	2	0.36	0.50	0.36	0.50
	3	0.36	0.36	0.36	0.69
	4	0.19	0.26	0.19	0.36
	5	0.36	0.69	0.69	0.69
7 days	1	0.20	0.08	0.20	0.12
	2	0.15	0.20	0.15	0.27
	3	0.15	0.08	0.08	0.08
	4	0.08	0.12		0.08
	5	0.08	0.15	0.27	0.08
10 days	1	0.08	0.08	0.08	0.12
	2	0.15	0.15	0.08	0.08
	3	0.15	0.07	0.07	0.08
	4	0.08	0.12	0.08	0.08
	5	0.05	0.05	0.04	0.05
14 days	1	0.08	0.15	0.08	0.15
	2	0.12	0.12	0.08	0.12
	3	0.07	0.08	0.08	0.12
	4	0.07	0.05	0.05	0.15
	5	0.05	0.15	0.08	0.04

III: Chemical Peritonitis

time	animal	biopsy number 1	2	3	4
6 hours	1	0.018	0.024	≤0.014	0.024
	2	0.024	≤0.014	0.032	0.024
	3	≤0.014	≤0.014	0.024	≤0.014
	4	0.018	0.043	0.024	0.043
	5	≤0.014	≤0.014	0.024	≤0.014
12 hours	1	≤0.014	0.018	≤0.014	0.018
	2	≤0.014	0.018	≤0.014	≤0.014
	3	≤0.014	≤0.014	≤0.014	≤0.014
	4	0.032	≤0.014	0.024	≤0.014
	5	≤0.014	0.024	≤0.014	≤0.014
1 day	1	0.050	0.050	0.050	0.098
	2	0.098	0.050	0.071	0.071
	3	0.050	0.050	0.050	≤0.027
	4	0.26	0.19	0.19	0.36
	5	0.050	0.098	0.098	0.050
4 days	1	0.36	0.36	0.36	0.36
	2	1.32	0.95	0.50	0.69
	3	0.69	0.69	0.69	0.95
	4	0.69	0.69	0.50	0.36
	5	0.50	1.32	1.32	1.32
7 days	1	0.47	0.27	0.47	0.20
	2	0.27	0.36	0.27	0.47
	3	0.47	0.27	0.47	0.63
	4	0.36	0.47	0.63	0.83
	5	0.27	0.09	0.27	0.20
10 days	1	0.20	0.36	0.27	0.63
	2	0.27	0.20	0.47	0.47
	3	0.09	0.05	0.36	0.15
	4	0.15	0.47	0.83	0.47
	5	0.47	0.36	0.47	0.63
14 days	1	0.63	0.47	0.63	0.47
	2	0.27	0.15	0.15	0.15
	3	0.36	0.47	0.47	0.20
	4	0.47	0.27	0.47	0.36
	5	0.63	0.47	0.47	0.27

IV: Peritoneal Ischaemia

time	animal	biopsy number 1	biopsy number 2
6 hours	1	0.05	0.05
	2	0.05	≤0.027
	3	0.071	0.05
	4	≤0.027	≤0.027
	5	0.05	0.071
12 hours	1	≤0.027	0.05
	2	0.098	0.05
	3	0.05	0.098
	4	0.05	≤0.027
	5	0.05	0.05
1 day	1	0.18	0.52
	2	0.89	0.30
	3	0.30	0.10
	4	0.30	0.035
	5	0.30	0.30
4 days	1	0.89	0.52
	2	0.52	0.30
	3	1.22	0.89
	4	0.89	-
	5	-	0.30
7 days	1	0.89	-
	2	0.52	1.52
	3	0.89	-
	4	0.89	-
	5	1.52	0.30
10 days	1	0.33	0.43
	2	0.15	-
	3	0.73	0.43
	4	-	0.43
	5	0.73	0.73
14 days	1	0.25	0.25
	2	0.25	0.25
	3	0.19	0.25
	4	0.25	0.15
	5	0.73	0.09
	6	0.15	0.25
	7	0.09	0.09
	8	0.09	0.09
	9	0.43	-
	10	0.11	0.09

Time Zero Controls

PAA (IU/cm²)

animal	biopsy number			
	1	2	3	4
1	0.126	0.166	0.075	0.075
2	0.126	0.219	0.126	-
3	0.126	0.126	0.075	0.126
4	0.042	0.042	0.075	0.042
5	0.042	0.042	0.075	0.075
6	0.075	0.126	0.126	0.126
7	0.126	0.126	0.219	0.095
8	0.126	0.075	0.075	0.075
9	0.166	0.126	0.075	-
10	0.042	0.126	0.042	0.042

Inflammation scores for experimental peritonitis

I: Bacterial Peritonitis

	time (days)						
	0.25	0.5	1	4	7	10	14
Acute features							
polymorphs	1	0	1	1	0	0	0
oedema	1	1	0	0	0	0	0
congestion	2	1	2	1	0	0	0
fibrin	0	0	0	0	0	0	0
Total	4	2	3	2	0	0	0
Chronic features							
fibrosis	0	1	0	1	0	0	0
chronic cells	0	1	1	1	1	1	0
granulation tissue	0	0	0	0	0	0	0
Total	0	2	1	2	1	1	0

II: Chemical Peritonitis

	time (days)						
	0.25	0.5	1	4	7	10	14
Acute features							
polymorphs	1	3	3	0	1	1	1
oedema	1	2	2	1	1	0	0
congestion	0	2	2	1	1	0	0
fibrin	0	0	1	0	0	0	0
Total	2	7	8	2	3	1	1
Chronic features							
fibrosis	0	0	0	0	2	3	3
chronic cells	0	1	2	1	2	2	2
granulation tissue	0	0	0	0	0	0	1
Total	0	1	2	1	4	5	6

III: Peritoneal Ischaemia

	time (days)						
	0.25	0.5	1	4	7	10	14
Acute features							
polymorphs	2	2	2	1	1	1	1
oedema	1	1	2	1	1	0	0
congestion	2	2	3	1	0	1	0
fibrin	2	1	1	1	0	0	0
Total	7	6	8	4	2	2	1
Chronic features							
fibrosis	0	0	0	2	1	3	3
chronic cells	0	0	1	2	2	3	3
granulation tissue	0	0	0	1	0	3	3
Total	0	0	1	5	3	9	9

Adhesion scores in peritoneal ischaemia group

animal	time (days) 0.25	0.5	1	4	7	10	14
1	4	2	5	3	5	6	5
2	2	5	5	6	3	5	6
3	3	4	6	4	6	5	5
4	5	5	5	5	6	4	3
5	3	4	3	5	5	5	6
median score	3	4	5	5	5	5	5

Microbiological assessment of bacterial peritonitis group

time	animal	bacterial count ($\geq 10^5$ /ml)			
		coliforms	staph	FTS	anaerobes
6 hours	1	+	+	-	-
	2	+	+	-	-
	3	+	+	-	-
	4	+	+	-	-
	5	+	+	-	-
12 hours	1	+	-	-	-
	2	-	-	-	-
	3	+	-	-	-
	4	-	+	-	-
	5	+	-	-	-
1 day	1	+	-	+	-
	2	+	-	+	-
	3	+	-	+	-
	4	+	-	+	-
	5	-	-	-	-
4 days	1	+	-	+	-
	2	-	-	-	-
	3	+	-	+	-
	4	-	-	-	-
	5	-	-	-	-
7 days	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-
10 days	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	+	-
14 days	1	-	-	-	-
	2	-	-	-	-
	3	-	-	+	-
	4	-	-	-	-
	5	-	-	-	-

APPENDIX CHAPTER 5

Control samples

Sample	peritoneal homogenate PAA (IU/cm^2)	homogenate plus goat serum PAA (IU/cm^2)
1	9.12	8.75
2	3.00	3.00
3	3.96	4.26
4	3.00	2.75
5	5.23	5.23
6	12.05	10.84
median	4.59	4.75

Antibody typing of human peritoneal PAA

Sample	PAA (IU/cm^2)	Dilution of antibody inhibiting activity by ≥50%	
		anti-tPA	anti-uPA
1	9.12	1:4096	1:4
2	3.00	1:4096	1:4
3	3.96	1:8192	1:4
4	3.00	1:4096	1:2
5	5.23	1:16384	1:8
6	12.05	1:32768	1:2

PAA and tPA levels in normal peritoneum

Sample	PAA (IU/cm^2)	tPA (ng/ml)
1	9.12	1.74
2	3.00	0.87
3	3.96	1.31
4	3.00	1.25
5	5.23	1.23
6	12.05	2.36
median	4.59	1.28

APPENDIX CHAPTER 6

Fibrinolytic parameters of normal and inflamed human peritoneum

I: Normal peritoneum

PAA (IU/cm^2)	tPA (ng/ml)	PAI-1 (ng/ml)	α_2-antiplasmin (μM/l)
11.5	1.34	≤2.5	≤0.05
6.5	0.32	≤2.5	≤0.05
4.7	0.83	≤2.5	≤0.05
13.9	1.36	≤2.5	≤0.05
14.8	1.44	≤2.5	≤0.05
12.5	1.05	≤2.5	≤0.05
8.3	0.74	≤2.5	≤0.05
17.7	2.10	≤2.5	≤0.05
2.1	0.67	≤2.5	≤0.05
22.9	1.01	≤2.5	≤0.05

II: Inflamed peritoneum

PAA (IU/cm^2)	tPA (ng/ml)	PAI-1 (ng/ml)	α_2-antiplasmin (μM/l)
0.10	0.40	6.7	≤0.05
0.07	1.02	21.0	≤0.05
0.10	0.15	8.1	≤0.05
0.07	≤0.05	2.7	≤0.05
0.42	1.30	8.8	≤0.05
≤0.04	1.20	9.7	≤0.05
0.07	1.08	24.1	≤0.05
0.07	0.92	10.4	≤0.05
0.34	0.67	6.5	≤0.05
≤0.04	1.20	4.84	≤0.05

APPENDIX CHAPTER 7

Fibrinolytic parameters of peritoneal exudate

fluid	patient	PAA (IU/ml)	tPA (ng/ml)	PAI-1 (ng/ml)
bile	1	0.339	14.54	≤2.5
	2	0.339	15.44	≤2.5
	3	0.525	5.18	≤2.5
	4	0.141	22.35	≤2.5
	5	0.815	17.81	≤2.5
serum	1	≤0.025	13.76	13.28
	2	≤0.025	13.02	20.68
	3	≤0.025	8.88	24.99
	4	≤0.025	8.79	36.05
	5	≤0.025	0.94	32.09
drain fluid	1	0.047	34.65	32.65
	2	≤0.025	30.46	27.37
	3	≤0.025	3.49	39.40
	4	≤0.025	18.68	41.30
	5	0.814	16.73	16.19

APPENDIX CHAPTER 8

Plasminogen activating activity of rtPA-gel

time (hr)	PAA of rtPA gel (IU/ml)			
	1500 cps	2250 cps	3000 cps	0.9% saline
2	≥4.25	≥4.25	≥4.25	≤0.084
4	≥4.25	≥4.25	≥4.25	≤0.084
6	≥4.25	≥4.25	≥4.25	≤0.084
8	≥4.25	≥4.25	≥4.25	≤0.084
10	≥4.25	≥4.25	≥4.25	≤0.084
12	≥4.25	≥4.25	≥4.25	≤0.084
14	≥4.25	≥4.25	≥4.25	≤0.084
16	≥4.25	≥4.25	≥4.25	≤0.084
18	≥4.25	≥4.25	≥4.25	≤0.084
20	≥4.25	≥4.25	≥4.25	≤0.084
22	≥4.25	≥4.25	≥4.25	≤0.084
24	≥4.25	≥4.25	≥4.25	≤0.084

Adhesion scores for the three treatment groups

	control	gel alone	rtPA-gel
	6	4	1
	3	2	3
	5	1	3
	5	2	0
	6	5	2
	3	3	0
	5	1	3
	4	3	0
	5	2	3
	6	2	1
	6	4	3
	4	3	0
median	5	2.5	1.5